Amendment Part of paper #20

Exhibit A

CURRICULUM VITAE

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Studies involve development of molecular binding assays for purified HLA class I and II MHC molecules. Research includes application of binding assays to characterizing specificity between MHC-peptide interactions. Experienced in size exclusion and reverse-phase HPLC. Current work has been focused on conversion of existing binding assays to a high throughput screening (HTS) platform.

Related work includes computer scanning of protein sequences for allele-specific, motif-bearing peptides. Additionally, work on the development of predictive algorithms (PIC) has been used to identify peptides with a given binding affinity potential.

Exhibit A (Southwood)

EPIMMUNE

TECHNICAL REPORT

Project: Epitope Identification **Departments:** Immunochemistry
Tumor Immunology

Tech. Report #: EDI-035-98

	Print Name	Signature	Date
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TITLE: Identification of HLA-A2 restricted epitopes for CEA, p53, MAGE2/3 and Her2/neu.

AUTHOR(S): Scott Southwood and Elissa Keogh

ABSTRACT:

HLA-A2 supertype degenerate peptides were identified from naturally occurring protein sequences of the tumor-associated antigens CEA, p53, Her2/neu, and MAGE2/3, utilizing the A2 supertype main anchor motif ($LIMVAT_{2,c}$) in conjunction with a customized computer program. Motif-positive peptides were synthesized and tested for binding capacity to purified A*0201, and high binders were then tested for binding to A*0202, A*0203, A*0206, and A*6802 MHC molecules. A total of 46 high binding and degenerate peptides were identified: ten CEA, ten p53, seventeen MAGE 2/3 and nine Her2/neu.

Exhibit B / Southwood Declaration

Modifying non-degenerate peptides by substituting anchor positions with canonical residues (L_2V_c), in an attempt to generate more degenerate binders, was also examined. Additional candidates which displayed increased HLA-A2 binding across all alleles were identified including (8) CEA, (12) p53, (5) MAGE2/3 and (8) Her2/neu.

Degenerate HLA-A2 supertype binders from each of the four antigens were evaluated for immunogenicity using an in vitro cellular assay. Some epitopes had been identified previously: CEA.691, CEA.605, CEA24V9, Her2/neu.435, Her2/neu.369, Her2/neu.5, MAGE3.112, MAGE3.157 and MAGE3. 271 [I. Kawashima, et al, Human Immunology 59(1):1-14, 1998]. Efforts described here have identified additional epitopes for MAGE2/3, CEA and p53. These include two overlapping MAGE epitopes, MAGE3.159 and MAGE3.160; three p53 epitopes, p53.139L2B3, p53,139L2 and p53.149M2; and three CEA epitopes, CEA.687, CEA.605V9 and CEA.233V10. Good population coverage was achieved for all four antigens as several CEA and MAGE2/3 epitopes demonstrate high binding affinity for all five alleles and the p53 and Her2/neu epitopes bind 4 of the 5 alleles. There are at least 2 epitopes for each tumor-associated antigen to consider for the preparation of a multi-epitope HLA-A2 cancer vaccine.

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INTRODUCTION:

The goal for this screening program was to identify at least 2 epitopes for the TAAs (tumor-associated antigens) MAGE2/3, Her2/neu, CEA (carcinoembryonic antigen) and p53 which bind at least three of the five most predominant HLA-A2 supertype alleles, and are able to induce CTLs that are peptide-specific, and recognize tumor targets endogenously expressing the antigen.

CEA is a 180kD cell surface and secreted glycoprotein produced by a number of different tumors, particularly colon cancer, but in a normal setting is associated with fetal tissue (Cancer Biology, 3rd ed., R. Ruddon, 1995:p 126). The abnormally high expression on cancer cells makes CEA an important target for immunotherapy.

MAGE, melanoma antigen genes, are a family of related proteins whose expression is normally limited to testis and placenta but is also expressed by melanomas and a variety of other carcinomas. These proteins are known to be recognized by cytotoxic T cells (Cellular and Molecular Immunology, 3rd ed., A. Abbas, A. Lichtman and J. Pober, 1997: p. 390).

Her2/neu or erbB-2 is a 185kD transmembrane protein that is similar to EGF receptor. The Her2/neu oncogene transforms cells when it is overexpressed and has tyrosine kinase activity making it capable of auto-phosphorylation. It is expressed primarily in breast, ovarian and gastric cancers (Cancer Biology, 3rd ed., R. Ruddon, 1995: p. 302).

The fourth TAA targeted, p53, is normally a tumor suppressor gene but when mutated, becomes tumorigenic and increases cell proliferation. The most common mutations are at positions 175, 248, 273 and 282 and these mutations have been observed in colon, lung, prostate and osteosarcomas as well as other tumors (Cancer Biology, 3rd ed., R. Ruddon, 1995: p. 323). These mutations result in increased expression of both the

wildtype and mutant p53 proteins, thereby qualifying this protein as a TAA.

Development of a multi-epitope vaccine should allow us to target colon, breast and lung carcinomas expressing these TAAs as well as having broader applications for the treatment of other carcinomas.

MATERIALS AND METHODS:

MHC sources.

The following EBV transformed cell lines were used as sources of class I molecules: JY (A*0201), FUN (A*0203), CLA (A*0206), and AMAI (A*6802). The mouse B cell lymphoma p815 transfected with A*0202 was also utilized.

Cells were maintained in vitro by culture in RPMI 1640 medium supplemented with 2mM L-glutamine and 10% heat-inactivated FCS. Cells were also supplemented with 100μg/ml of streptomycin [Irvine Scientific, Santa Ana, CA] and 100U/ml of penicillin [GIBCO, Grand Island, NY]. Large quantities of cells were grown in roller bottles.

Affinity purification of HLA-A molecules.

Cells were lysed at a concentration of 10^8 cells/ml in PBS containing 1% NP-40 and 1mM PMSF (phenylmethylsulfonyl fluoride, Sigma P-7626, St. Louis, MO). The lysates were cleared of debris and nuclei by centifugation at 10,000 x *g* for 20min.

MHC molecules were then purified by affinity chromatography as previously described (A. Sette et al, J Immunol 142:35, 1989). Columns of inactivated Sepharose CL4B and Protein A Sepharose were used as pre-columns. Lysates were filtered through 0.8 and 0.4μM filters and then depleted of HLA-B and HLA-C molecules by repeated passage over Protein A Sepharose beads conjugated with the anti-HLA(B,C) antibody B1.23.2 (N. Rebai et al, Tissue Antigens 22:107, 1983). Typically 2 to 4 passages were required for

effective depletion. Subsequently, the anti-HLA(A,B,C) antibody W6/32 (C. J. Barnstable et al, Cell 14:9, 1978) was used to capture HLA-A molecules.

Independently, both antibody columns were washed with 15-column volumes of 10mM TRIS in 1.0% NP-40, PBS and 2-column volumes of PBS containing 0.4% n-octylglucoside. Finally, the class I molecules were eluted with 50mM diethylamine in 0.15M NaCl containing 0.4% n-octylglucoside, pH 11.5. A 1/25 volume of 2.0M Tris, pH 6.8, was added to the eluate to reduce the pH to ~8.0, and then concentrated by centifugation in Centriprep 30 concentrators (Amicon, Beverly, MA) at 2000rpm. Protein purity, concentration, and effectiveness of depletion steps were monitored by SDS-PAGE.

A2 supertype molecular binding assays.

Purified human class I molecules [5 to 500nM] were incubated with 1-10nM ^{125}I-radiolabeled probe peptide, iodinated by the Chloramine T method (S. Buus et al, Science 235:1335, 1987), for 48h at room temperature in the presence of 1 μM human $ß_2$ microglobulin (Scripps Laboratories, San Diego, CA) and a cocktail of protease inhibitors. The final concentrations of protease inhibitors were: 1mM PMSF, 1.3nM 1.10 phenanthroline, 73μM pepstatin A, 8mM EDTA, and 200μM N alpha-tosyl-lysine chloromethyl ketone (TLCK).

Class I peptide complexes were separated from free peptide by gel filtration on TSK200 columns, and the fraction of bound peptide calculated as previously described (A. Sette et al, J Immunol 142:35, 1989). In preliminary experiments, the HLA class I prep was titered in the presence of fixed amounts of radiolabeled peptides to determine the concentration of class I molecules necessary to bind 10-20% of the total radioactivity. All subsequent inhibition and direct binding assays were then performed using these class I concentrations. In the inhibition assays, peptide inhibitors were typically tested at

concentrations ranging from 120μg/ml to 1.2ng/ml. The data were then plotted and the dose yielding 50% inhibition was measured. Peptides were tested in two to four completely independent experiments. Since under these conditions [label]<[MHC] and IC50≥[MHC], the measured IC50s are reasonable approximations of the true k_D values.

Radiolabeled probe and standard control peptides used are as follows:

A $F_6 \rightarrow Y$ analog of the HBV core 18-27 peptide (sequence FLPSDYFPSV; J. Ruppert et al, Cell 74:929, 1993) was used as the radiolabeled probe for the A*0201, A*0202, A*0203, and A*0206 assays. A $C_4 \rightarrow A$ analog of the HBV pol 646-654 peptide (sequence FTQAGYPAL) was used as the radiolabeled probe for the A*6802 assay. The average IC50's of $F_6 \rightarrow Y$ analog of the HBV core 18-27 peptide for the A*0201, A*0202, A*0203, and A*0206 assays were 5.0nM, 4.3nM, 10nM, and 3.7nM, respectively. The average IC50 of the $C_4 \rightarrow A$ analog of the HBV pol 646-654 peptide in the A*6802 assay was 8.0nM.

Peptide Synthesis.
Peptides were either synthesized at Cytel Corporation (San Diego, CA), as previously described (J. Ruppert et al, Cell 74:929, 1993); or, for large epitope libraries, purchased as crude material from Chiron Technologies (Chiron Corp., Clayton, Victoria, Australia). Peptides synthesized at Cytel were purified to >95% homogeneity by reverse-phase HPLC. The purity of these synthetic peptides was determined using an analytical reverse-phase column and their composition confirmed by amino acid analysis and/or mass spectrometry analysis.

Target Cell Lines for Cellular Screening. The .221A2.1 cell line, produced by transferring the HLA-A2.1 gene into the HLA-A, -B, -C null mutant human B-lymphoblastoid cell line .221, was used as the peptide-loaded target to measure activity of HLA-A2.1 restricted CTL. The HLA-typed melanoma cell lines (624mel and 888mel)

were a generous gift from Y. Kawakami and S. Rosenberg, National Cancer Institute, Bethesda, MD. The colon adenocarcinoma cell lines SW403 and HT-29, the osteosarcoma line Saos-2 and the breast tumor line BT549 were obtained from the American Type Culture Collection (Rockville, MD). The gastric cancer line , KATO III, was obtained from the Japanese Cancer Research Resources Bank. The Saos-2/175 (Saos-2 transfected with the p53 gene containing a mutation at position 175) was obtained from Dr. Levine, Princeton University, Princeton, NJ. All cell lines, except those from the ATCC, were grown in RPMI-1640 medium supplemented with antibiotics, sodium pyruvate, nonessential amino acids and 10% (v/v) heat inactivated FCS. The melanoma, colon and gastric cancer cells were treated with 100U/ml IFNγ (Genzyme) for 48 hours at 37°C before using them as targets in the ^{51}Cr release and in situ IFNγ assays. The p53 tumor targets were treated with 20ng/ml IFNγ and 3ng/ml TNFα for 24 hours prior to assay (M. Theobald, et al PNAS 92:11993, 1995).

Primary CTL Induction Cultures.

Generation of Dendritic Cells (DC): PBMCs were thawed in RPMI with 30μg/ml DNAse, washed twice and resuspended in complete medium (RPMI-1640 plus 5 % AB human serum, non-essential amino acids, sodium pyruvate, L-glutamine and penicillin/streptomycin). The monocytes were purified by plating 10 X 10^6 PBMC in 3 ml of complete medium in each well of a 6-well plate. After 2 hrs at 37°C, the non-adherent cells were removed by gently shaking the plates and aspirating the supernatants. The wells were washed a total of three times with 3 ml RPMI to remove most of the non-adherent and loosely adherent cells. Three 3 ml of complete medium containing 50 ng/ml of GM-CSF and 1,000U/ml of IL-4 were then added to each well and the DC were ready to be used for CTL induction cultures after 7 days.

Induction of CTL with DC and Peptide:

CD8+ T-cells were isolated by positive selection with Dynal immunomagnetic beads and

detachabead reagent according to the manufacturer's instructions. Typically 200~250x10^6 PBMC were processed to obtain 24x10^6 CD8+ T cells (enough for a 48-well plate). Briefly, the PBMCs were thawed in RPMI with 30µg/ml DNAse, washed once with PBS containing 1% human AB serum and resuspended in PBS/1% AB serum at a concentration of 20x10^6cells/ml. The magnetic beads were washed 3 times with PBS/AB serum, added to the cells (140µl beads/20x10^6 cells) and incubated for 1 hour at 4°C with continuous mixing. The beads and cells were washed 4x with PBS/AB serum to remove the nonadherent cells and resuspended at 100x10^6 cells/ml (based on the original cell number) in PBS/AB serum containing 100µl/ml detacha-bead reagent and 30µg/ml DNAse. The mixture was incubated for 1 hour at room temperature with continuous mixing. The beads were washed again with PBS/AB/DNAse to collect the CD8+ T-cells.

The DC were collected and centrifuged at 1300 rpm for 5-7 minutes, washed once with PBS with 1% BSA, counted and pulsed with 40µg/ml of peptide at a cell concentration of 1-2x10^6/ml in the presence of 3µg/ml β_2- microglobulin for 4 hours at 20°C. The DC were then irradiated (4,200 rads), washed 1 time with medium and counted again.

Setting up induction cultures: 0.25 ml cytokine-generated DC (@1x10^5 cells/ml) were co-cultured with 0.25ml of CD8+ T- cells (@2x10^6 cell/ml) in each well of a 48-well plate in the presence of 10 ng/ml of IL-7. rHuman IL10 was added the next day at a final concentration of 10 ng/ml and rhuman IL2 was added 48 hours later at 10IU/ml.

Restimulation of the induction cultures with peptide pulsed adherent cells: Seven and fourteen days after the primary induction, the cells were restimulated with peptide-pulsed adherent cells. The PBMCS were thawed and washed twice with RPMI and DNAse. The cells were resuspended at 5x10^6 cells/ml and irradiated at ~4200 rads. The

PBMCs were plated at $2x10^6$ in 0.5 ml complete medium per well and incubated for 2 hours at 37°C. The plates were washed twice with RPMI by tapping the plate gently to remove the nonadherent cells and the adherent cells were pulsed with 10 μg/ml of peptide in the presence of 3 μg/ml β_2 microglobulin in 0.25ml RPMI/5%AB per well for 2 hours at 37°C. Peptide solution from each well was aspirated and the wells were washed once with RPMI. Most of the media was aspirated from the induction cultures (CD8+ cells) and brought to 0.5 ml with fresh media. The cells were then transferred to the wells containing the peptide-pulsed adherent cells. Twenty four hours later rhuman IL10 was added at a final concentration of 10 ng/ml and rhuman IL2 was added the next day and again 2-3 days later at 50IU/ml (V. Tsai, Critical Reviews in Immunology 18(1-2):65-75, 1998). Seven days later the cultures were assayed for CTL activity in a ^{51}Cr release assay. In some experiments the cultures were assayed for peptide-specific recognition in the in situ IFNγ ELISA at the time of the second restimulation followed by assay of endogenous recognition 7 days later. After expansion, activity was measured in both assays for a side by side comparison.

Measurement of CTL lytic activity by ^{51}Cr release.

Seven days after the second restimulation, cytotoxicity was determined in a standard (5 hr) ^{51}Cr release assay by assaying individual wells at a single E:T. Peptide-pulsed targets were prepared by incubating the cells with 10μg/ml peptide overnight at 37°C. Adherent target cells were removed from culture flasks with trypsin-EDTA. Target cells were labelled with 200μCi of ^{51}Cr sodium chromate (Dupont, Wilmington, DE) for 1 hour at 37°C, washed twice and resuspended at 10^6 per ml and diluted 1:10 with K562 cells (an NK- sensitive erythroblastoma cell line used to reduce non-specific lysis) at a concentration of $3.3x10^6$/ml . Target cells (100 μl) and 100μl of effectors were plated in 96 well round-bottom plates and incubated for 5 hours at 37°C. At that time, 100 μl of supernatant were collected from each well and percent lysis was determined according to the formula: [(cpm of the test sample - cpm of the spontaneous ^{51}Cr release

sample)/(cpm of the maximal ^{51}Cr release sample - cpm of the spontaneous ^{51}Cr release sample)] x 100. Maximum and spontaneous release were determined by incubating the labelled targets with 1% Trition X-100 and media alone, respectively. A positive culture was defined as one in which the specific lysis (sample - background) was 10% or higher in the case of individual wells and was 15% or more at the 2 highest E:T ratios when expanded cultures were assayed.

In situ Measurement of Human γIFN Production as an Indicator of Peptide-specific and Endogenous Recognition.

1. The day before the assay, 96-well Immulon 2 plates were coated with 50µl/well mouse anti-human γIFN monoclonal antibody (Pharmingen 18891D) prepared at 4µg/ml of coating buffer (0.1M $NaHCO_3$, pH8.2) and incubated overnight at 4°C. One plate was set up for each target to be tested.
2. The plates were washed 4x with wash buffer [PBS (Ca^{2+} and Mg^{2+} free) with 0.05% Tween 20], 200µl blocking solution (PBS with 10% FCS) was added and the plates were incubated for 2 hours at room temperature.
3. The plates were washed 3x with wash buffer and 1x with PBS.

4a. The CTLs (100µl/well) and targets (100µl/well) in media (RPMI/5% human AB serum/pen-strep/glutamine/nonessential aa/Na pyruvate) were added to each well, leaving empty wells for the standards and blanks (which received media only). For expanded cultures, $1x10^5$ CTL/well are then mixed with $1x10^5$ targets (neg. control) or peptide-pulsed targets or endogenous targets. All wells were brought to 200µl with medium and incubated for 48 hours at 37°C with 5% CO_2.

4b. The media was removed from the standard wells and recombinant human γIFN was added starting at 400 pg/100ul/well, followed by 4 3-fold dilutions. The plate was incubated for 2 hours more at 37°C.

5. The plates were then washed 4x with wash buffer and 100µl biotinylated mouse anti-human γIFN monoclonal antibody (Pharmingen 18902D) prepared at

4μg/ml of diluent (PBS with 3% FCS and 0.05% Tween 20) was added to each well and the plates incubated for 2 hours at room temp.

7. The plates were washed 4x with wash buffer and 100μl/well HRP-streptavidin (Zymed, 43-4323, 1:4000 in diluent) was added and incubated for 1 hour at room temp.

9. The plates were then washed 6x with wash buffer, 100μl/well developing solution (TMB 1:1) was added and developed for about 15 min. The reaction was stopped with 50 ul/well 1M H_3PO_4 and read at OD450.

A culture was considered positive if it measured at least 50 pg of γIFN/well and was twice the background level of expression.

CTL Expansion. Those cultures that demonstrated specific lytic activity against peptide-pulsed targets and/or tumor targets were expanded over a two week period with anti-CD3. Briefly, $5x10^4$ CD8+ cells were added to a T25 flask containing the following: $1x10^6$ irradiated (4,200 rad) PBMC (autologous or allogeneic) per ml, $2x10^5$ irradiated (8,000 rad) EBV- transformed cells per ml, and OKT3 at 30 ng per ml in RPMI-1640 containing 10% (v/v) human ab serum, non-essential AA, sodium pyruvate, 25μM 2-ME, L-glutamine and penicillin/streptomycin. rHuman IL2 was added 24 hours later at a final concentration of 200IU/ml and every 3-4 days thereafter with fresh media at 50 IU/ml. The cells were split if the cell concentration exceeded $1x10^6$/ml and the cultures were assayed between days 13 and 15 at 4 E:T ratios (30, 10, 3 and 1:1) using the same targets as before the expansion.

RESULTS AND DISCUSSION:

1. Peptides identified from wild-type (WT) sequences.

Identification of HLA-A2 supertype degenerate peptides.

Protein sequences from the targeted four tumor antigens (p53, CEA, Her2/neu, and

MAGE2/3) were scanned, utilizing a customized program, to identify 8-, 9-, 10-, and 11-mer sequences containing the HLA-A2 supertype main anchor motif. That motif is leucine (L), isoleucine (I), valine (V), methionine (M), alanine (A), or threonine (T) at both position 2 and the C-terminus. Nonamer and decamer sequences were further characterized by evaluating the presence of A2 preferred secondary anchor residues (Ruppert, et al, Cell 74:929,1993) by use of an A2-specific algorithm. The criteria used to select sequences for peptide synthesis varies between targets, and is addressed in each target section separately.

All peptides were tested for their capacity to bind purified HLA-A*0201 molecules in vitro. Peptides exhibiting high or intermediate affinity were considered further. High binding affinity is defined as a 50% inhibitory concentration (IC_{50}) of ≤50nM. Intermediate binders are defined as having IC_{50}s in the 50-500nM range.

A*0201 "binders" were then tested on other predominant molecules of the A2 supertype family (A*0202, A*0203, A*0206, and A*6802) (Rev: Sidney, et al, Immunology Today 17:291, 1996). Peptides which are degenerate A2 supertype binders (binding at least three of the five alleles tested) were classified as "crossbinders", and candidates for cellular screening analysis.

A peptide was considered to be an epitope if it induced peptide-specific CTLs in at least 2 donors (unless otherwise noted) and if those CTLs also recognized the endogenously expressed peptide.

Molecular binding of wild-type CEA-derived peptides.

Following the strategy outlined above, 336 WT A2 motif-positive sequences were identified within the CEA protein sequence. Of these, 266 peptides were synthesized and tested for A*0201 binding affinity.

Fourteen of these 266 peptides bound A*0201 with high or intermediate affinity and have been tested for A2 crossbinding capacity (NB 903, p. 38). Ten of the 14 peptides tested against A2 supertype molecules bound 3 or more A2 supertype alleles (Table I).

Identification of HLA-A2 restricted CEA-derived epitopes.

Ten peptides have been identified as supertype binders for HLA-A2. Nine peptides were tested in the cellular assay and 6 of these peptides were able to induce a peptide-specific CTL response in at least 2 normal donors except as noted. Previous work done by our group and others demonstrated that additional restimulations were required to detect endogenous recognition when CTLs were induced with CEA peptides. Cultures that lysed peptide-pulsed targets were restimulated twice more before assaying for endogenous recognition. This analysis demonstrated that 5 of these lines also recognized target cells pulsed with the wild-type peptide and tumor targets that endogenously express CEA (Table II).

The epitopes CEA.691 and CEA.605 had been identified previously [I. Kawashima, et al, Human Immunology 59(1):1-14, 1998; E. Ras et al, Human Imm 53:81, 1997; K. Tsang et al, Clin. Cancer Res. 3:2439, 1997, S. Zaremba, et al, Cancer Res. 57:4570, 1997] and so recent efforts have concentrated on CEA.233, CEA.78, CEA.569 and CEA.687. Peptide specific CTLs to CEA.233 (NB 888, p.148), CEA.569 (NB 888, p.179) and CEA. 687 (NB 888, p.171) were observed in one to two donors but endogenous recognition was observed only with CEA.687. Figure 1 shows the 15 wells that demonstrated a positive response to CEA.687 in a ^{51}Cr release assay.

CTLs from each well were expanded and reassayed against peptide-pulsed and endogenous targets. The results of 4 individual cultures are shown in Figures 2a, 2b, 2c and 2d. As can be seen in 2a through 2c, the peptide-specific CTLs also recognize the endogenous targets. One culture (Fig. 2d) demonstrates significant lysis of peptide-

pulsed targets but not tumor targets. The CTLs shown in Figures 2a and 2b were also tested against 221A2.1 target cells pulsed with different concentrations of peptide to measure CTL avidity. One line demonstrated high specific lysis down to 1 ng/ml peptide while both exhibited a titration of activity further validating CEA.687 as an epitope (Figure 3).

Even more convincing is the cold target inhibition assay (Figure 4) in which peptide-pulsed targets were incubated with ^{51}Cr labelled targets to compete for lysis by the CTLs. As can be seen, lysis of radiolabelled targets by two different CTL lines was blocked by increasing numbers of targets pulsed with CEA.687. When an irrelevant peptide was used, in this case HBVc.18, no inhibition of lysis was seen again demonstrating the epitope specificity of these CTLs.

Molecular binding of wild-type p53-derived peptides.

133 sequences carrying the A2 supertype motif were identified within the p53 protein sequence, and all were synthesized. Through the course of previous binding studies, peptides which carry glutamine (Q) at position 2 (L, I, V, M, A, or T at the C-terminus) have also been shown to possess some capacity to bind A2-like molecules. Sixteen Q_2 peptides were also synthesized. Herein, we report that 14 of these 149 peptides bound A*0201 with high or intermediate affinity (NB 903, pp.24, 43).

These 14 peptides were then tested for A2 crossbinding capacity and the results are presented in Table III. Ten highly crossreactive peptides were identified. Although nine of these were disclosed in the C. Melief application (filed internationally May 18, 1993) and by H. Nijman, et al, Immunol. Lett. 40:171, 1994, one WT peptide (position 135, CQLAKTCPV) was not. To avoid any potential issues with the Melief application only the p53.135 peptide was pursued further.

Identification of HLA-A2 restricted p53-derived epitopes.

One undisclosed p53-derived peptide (p135) was identified as a supertype binder and this peptide induced peptide-specific CTLs in only one donor while endogenous recognition was not observed (Table IV, NB 1002, p. 81).

Molecular binding of wild-type MAGE2/3-derived peptides.

285 WT peptides were identified within the MAGE2 and/or the MAGE3 protein sequence. Of these, 137 have been tested for A*0201 binding affinity, with 19 binders being identified (NB 903, p.42). Seventeen of the 19 A*0201 binders are degenerate A2 supertype binders (Table V).

Identification of HLA-A2 restricted MAGE2/3-derived epitopes.

Motif analysis and binding studies identified seventeen potential epitopes for both MAGE2 and 3. However, four peptides are identical in both MAGE2 and 3, and therefore do not all represent distinct epitopes. A total of 13 peptides were screened in the cellular assay and 9 peptides were able to induce a response in PBMCs from at least 2 normal donors. CTLs to 5 of these peptides were also able to recognize endogenously expressed peptide (see Table VI). Two of these peptide sequences, MAGE3.159 and MAGE3.160, overlap and while both bind to 5 alleles, MAGE3.160 binds with a higher affinity to 4 of the 5 alleles. Figures 5a and 5b show the results of the γIFN in situ ELISA of individual CTL cultures induced with MAGE3.159 (NB 1002, p.134). Cells from five wells recognized the peptide-pulsed targets (Figure 5a) and 2 of these wells also recognized the appropriate tumor target (Figure 5b). Additionally. MAGE3.160 induced a peptide-specific CTL response in 14 out of 48 wells (Figure 6a) and 3 of these wells demonstrated endogenous recognition in the γIFN assay (Figure 6b, NB 1002, p. 134).

MAGE3.112 is also an excellent candidate because of its binding and immunogenicity [I. Kawashima, et al, Human Immunology 59(1):1-14, 1998; M. Visseren, Int. J.

Cancer,73(1):125, 1997]. MAGE2.157 (M. Visseren, Int. J. Cancer,73(1):125, 1997) and MAGE3.271 have also been identified as epitopes [I. Kawashima, et al, Human Immunology 59(1):1-14, 1998].

Molecular binding of wild-type Her2/neu-derived peptides.

623 WT peptides have been identified within the Her2/neu protein sequence. Of these, 73 scored positive in the A2 algorithm and were synthesized. An additional 90 nonamers and decamers were also synthesized. These 163 were tested for A*0201 binding affinity, with 20 binders being identified (NB 903, p. 40).

All 20 A*0201 binders have been tested for A2 supertype alleles, 9 of them possessing crossbinding capability (Table VII).

Identification of HLA-A2 restricted Her2/neu-derived epitopes.

A total of 9 peptides were identified as candidates for cellular screening by analysis of the Her2/neu sequence. The results of the cellular screening assays are shown in Table VIII [I. Kawashima, et al, Human Immunology 59(1):1-14, 1998]. As can be seen, two peptides, Her2/neu.435 and Her2/neu.369 (B. Fisk et al, J. Exp. Med. 181:2109, 1995), induced CTLs in at least two donors and both recognized tumor targets as well. An example of Her2/neu.369 is shown in figure 7 (NB 888, p. 61). An additional wild-type peptide, Her2/neu.5 was screened on the basis of its A2.1 binding and although it binds to only 2 alleles, it was able to generate a strong CTL response that was both peptide- and tumor-specific.

Additional information on many of the same peptides was generated by a collaborator, Dr. Rolf Kiessling, (Table VIII; Y. Rongcun, Y., et al, Identification of new HER2/neu derived peptide epitopes which can elicit CTL cytotoxic for autologous and allogeneic

carcinomas, in preparation.). Briefly, PBMCs were isolated from two patients with ovarian cancer, restimulated with peptide-pulsed monocytes and assayed for their ability to recognize peptide-pulsed target cells as well as transfected cells endogenously expressing the antigen. Based on this data, Her2/neu.435 is recognized in 2 donors as well as Her2/neu.369, Her2/neu.952 and Her2/neu.48. Her2/neu.689 is also an epitope but not a supertype binder. Of the other peptides tested, Her2/neu.665 and Her2/neu.773 were recognized by CTLs from only one of the two patients and CTLs to Her2/neu.153 and Her2.neu.789 recognized peptide-pulsed targets alone.

2. Fixed peptides.

Identification of HLA-A2 supertype degenerate fixed peptides.

It has been shown that class I peptide ligands can be modified, or "fixed" to increase their binding affinity and/or degeneracy (Sidney et al, J Immunol 157:3480; Rosenberg, et al, Nature Med. 4:321, 1998). As a supplemental strategy to ensure adequate numbers of degenerate A2 binders for cellular screening, we explored "fixing" peptides at primary anchor positions. Peptides exhibiting at least weak A*0201 binding (IC_{50} of ≤5000nM), and carrying suboptimal anchor residues at either position 2, C termini, or both, can be fixed by introducing canonical substitutions (L at position 2 and V at the C-terminus).

Fixed analog peptides which show at least a three-fold increase in A*0201 binding and bind minimally with an IC_{50} of 500nM, were then tested for A2 crossbinding capacity, along with their wild-type (WT) peptide counterparts. As with WT peptides, fixed analogs must bind at least three of the five A2 supertype alleles to be considered for cellular screening.

Additionally, the selection of analogs for cellular screening was further restricted by their WT parent peptide's capacity to crossbind at least weakly, that is, to have an IC_{50} of

≤5000nM for 3 or more A2 alleles. The rationale for this requirement is that the WT peptide must be present endogenously in sufficient quantity to be biologically relevant. Fixed peptides have been shown to have increased immunogenicity and cross reactive recognition by T cells specific for the WT epitope (Parkhurst et al, J Immunol 157:2539; Pogue et al, PNAS 92:8166).

Another form of fixing instituted in these studies, unrelated to the primary anchor position, involves the substitution of a cysteine (C) for α-amino butyric acid (B). Due to its chemical nature, cysteine has the propensity to form disulfide bridges and sufficiently alter the peptide structurally as to reduce binding capacity. Substituting B for C not only alleviates this problem, but has been shown to actually improve binding and crossbinding capability in certain instances (Review: A. Sette et al, Persistent Viral Infections, Ed. R. Ahmed and I. Chen, John Wiley & Sons, England).

In the cellular screening of these peptide analogs, it was important to demonstrate that analog-specific CTLs were able to recognize the wild-type peptide and when possible, tumor targets endogenously expressing the epitope.

Molecular binding of CEA fixed peptides.

Sixty-five peptides met the criteria to be fixed by introducing canonical substitutions, showing at least weak A*0201 binding (IC_{50} ≤5000nM) and carrying suboptimal anchor residues.

To date, 10 analogs of 9 peptides have been fixed, and both analog and WT versions were tested for crossbinding capacity on other A2 supertype molecules (Table IX).

Eight of the 10 analogs bound minimally 3 of 5 A2 supertype alleles, and their WT versions also bound at least weakly to 3 of 5 alleles (NB 903, p.38). An exception to the

general strategy was made in the case of the CEA.605 peptide, insomuch that the fixed analog does not have a three-fold increase in A*0201 binding affinity. This peptide does, however, have increased crossbinding capability, and therefore was included in the list of peptides to be examined in the cellular screening assay.

Cellular screening of CEA analogs.

Eight analogs were identified for cellular screening. One of these, CEA.24V9, was previously identified as an epitope [Kawashima, I. et al, Human Immunology 1998; Jan: 59(1):1-14]. Three additional peptides were screened and, as shown in Table X, CEA.233V10 (NB 888, pp. 60, 101 and 148), CEA.605V9 (NB 888, p. 148) and CEA.589V9 (NB 888, p. 162) all induced CTL that were able to recognize peptide-pulsed and/or tumor targets after 2 and 4 restimulations respectively. Figures 8a and 9a are examples of the peptide specific lysis seen with peptides CEA.233V10 and CEA.605V9 respectively. After expansion of the positive cultures, the CTLs were again tested against the analog and also the parental peptide and tumor targets (Figures 8b and 9b). CTLs to both analogs demonstrated recognition of the wildtype peptide and the tumor line, KATO III. In addition to being immunogenic, CEA.233V10 and CEA.605V9 showed improved overall binding when compared to the corresponding wildtype peptide as well as supertype binding to 4 alleles. An additional epitope, CEA.589V9, was immunogenic and CEA.589V9-specific CTLs recognized the wildtype peptide (Figures 10a, 10b and 10c) but endogenous recognition was not observed.

Molecular binding of p53 fixed peptides.

Nineteen peptides meeting the prescribed parameters were fixed (NB 903, p. 25). The results are shown in Table XI.

Eighteen analog peptides which meet the prescribed criteria were tested for crossbinding capacity on other A2 supertype molecules (Table XII). Eleven of these analogs exhibited

improved crossbinding capability (NB 903, p. 57).

Cellular screening of p53 analogs.

Eleven analogs have been identified as HLA-A2 supertype binding peptides (Table XIII) and cellular screening was done with all 11 peptides. In the case of p53, it was important to demonstrate induction of peptide-specific CTL and then to use those cells to identify an endogenous tumor target. Each assay also included the epitope HBVc.18 as an internal control. When peptide p53.139L2 was used to induce CTLs in a normal donor, measurable CTL activity was observed in 3 of 48 wells (Figure 11a, NB 888, p. 79). Each well was expanded and two weeks later reassayed against the induction peptide and the appropriate wildtype peptide. As can be seen, p53.139L2-specific CTLs expanded well and maintained their lytic activity. Additionally, two of these cultures recognized the parental, wildtype peptide (Figures 11b and 11c, NB 888, p. 139).

These cells were then used to assess endogenous target cell lines. Numerous HLA-A2+, p53 expressing tumor lines have been described in the literature (W. Storkus, personal communication; M. Theobald et al, PNAS 92: 11993, 1995) and were readily available. These included MCF-7, a breast tumor line; BT549, breast, infiltrating ductal carcinoma and SW480, colon adenocarcinoma (PNAS 1995, 92: 11993). Saos-2, an osteogenic sarcoma which is HLA-A2+ and p53- was used as the negative endogenous target. Figures 12a and 12b show that two individual CTL cultures to peptide p53.139L2 demonstrated significant lysis of the endogenous target BT549 (NB888, p. 139). This was the target line used for all subsequent assays. We were also able to obtain the Saos-2/175 line (Saos-2 transfected with the p53 gene) at a later time and this line was also included as a tumor target as noted.

Of the available analogs tested, ten induced a peptide-specific response in 2 or more donors. Of these 10, eight generated CTLs that recognized the wild-type peptide and 4 of

these recognized tumor targets (Table XIII). Two of these analogs, p53.139L2 and p53.139L2B3 , differ only at position three. Figures 13a and 13b (NB 888, p.140) show the assay results of CTLs to p53.139L2B3 tested against peptide-pulsed and tumor targets. As can be seen in Figure 13b, these CTLs recognize the wild-type peptide as well as the analog and tumor target BT549. p53.149M2, an analog that represents a significant improvement over the wildtype peptide is shown in Figures 14a and 14b (NB 888, p. 152). Six individual wells met the criteria for positivity and one well maintained that activity upon expansion. All the CTLs generated recognized the wildtype peptide and were also able to lyse the Saos-2/175 transfected line. A fourth epitope, p53.69L2V8, also demonstrated recognition of the wildtype peptide (data not shown, NB 888, p. 151).

Molecular binding of MAGE2/3 fixed peptides.

Of the 19 MAGE2/3-derived A*0201 binders, 14 carry suboptimal anchors and are candidates for anchor fixing. To date, 5 analogs of two WT peptides have been synthesized and tested for A2 supertype molecules (NB 903, p. 58; Table XIV).

MAGE3.112 analogs exhibit increased A*0201 binding affinity, but the parent peptide already binds all 5 A2 supertype alleles, and significant improvement in this area was not achieved. The MAGE3.220 analog, however, does meet all fixed analog criteria, having increased A*0201 binding affinity three-fold and improved crossbinding.

Additionally, 24 of 26 weak binding A*0201 peptides are also candidates.

Cellular screening of MAGE2/3 analogs.

No cellular screening was done because the analogs provided no improvement over the wild-type.

Molecular binding of Her2/neu fixed peptides.

Of the 20 A*0201 binders, 15 carry suboptimal anchors, and are candidates for fixing. To date, 8 analogs of 6 A*0201 binders have been fixed (NB 903, p.58), and tested for primary and supertype binding (Table XV). In five of 6 cases, A*0201 binding was improved at least three-fold. In three cases, crossbinding capability was also improved. In one special case, peptide Her2/neu.153 did not have a three-fold increase in A*0201 binding, but crossbinding capability was improved.

Additionally, 22 weak binding A*0201 peptides carrying suboptimal anchors were also identified, and can be fixed if desired.

Cellular screening of Her2/neu analogs.

Two analogs of Her2/neu.5 and 1 version of Her2/neu.952 were screened in the primary CTL induction assay. Her2/neu.5B3V9 (NB 865, pp. 148, 168) and Her2/neu.952L2B7V10 (NB 865, pp. 143, 148, 170) induced peptide-specific CTLs in at least 2 donors (data not shown) but when the positive cultures were expanded, no wild-type peptide or endogenous recognition was observed (Table XVI).

CONCLUSION:

For each of the four tumor antigens CEA, p53, Her2/neu, and MAGE2/3, at least 2 peptides were identified that satisfy each of the parameters that define an epitope: 1) they are degenerate A2 supertype binders, possessing IC_{50}s ≤500nM for three of the five A2 supertype alleles tested, 2) they elicit a peptide-specific CTL response and 3) they recognize the wild-type peptide and/or tumor targets.

The HLA-A2 restricted epitopes identified by antigen are as follows:

CEA: CEA687, and CEA691.

MAGE2/3: MAGE2.157, MAGE3.112, and MAGE3.160.

Her2/neu: Her2/neu.369 and Her2/neu.435.

As a footnote, Her2/neu.5 and Her2/neu.665 should be given consideration as possible clinical candidates because they both elicit good cellular response and have endogenous killing, even though both fail to bind more than 2 A2 supertype alleles.

To supplement the previously described epitopes, 6 unique epitopes were identified when peptides derived from CEA and p53 were fixed to improve binding and increase the number of alleles to which each bound. These include:

CEA: CEA.24V9, CEA233.V10 and CEA605V9.

p53: p53.139L2, p53.139L2B3, and p53.149M2.

Figure legends

Figure 1a. Recognition of CEA.687 peptide-pulsed targets by peptide specific CTLs. CTLs from individual wells were screened against 221A2.1 without peptide (hatched bars) or 221A2.1 pulsed overnight with 10 ug/ml peptide (solid bars). K562 cells were added to reduce lysis by natural killer (NK) cells.

Figures 2a-2d. Expanded CEA.687 specific CTLs from wells 15, 23, 42, and 41 respectively recognize peptide-pulsed and tumor targets. The CTLs were tested against 221A2.1 cells (open circle), 221A2.1 pulsed with CEA.687 (closed circle), HT29, the negative endogenous target (open square) and SW403, the CEA positive tumor target (closed square).

Figure 3. CEA.687 peptide concentration affects lysis of target cells by peptide- specific CTLs. Expanded CEA.687 specific CTLs from wells 15 (open circles) and 23 (closed squares) were tested against 221A2.1 target cells pulsed overnight with varying amounts of peptide.

Figure 4. Lysis of radiolabelled endogenous targets by CEA.687 specific CTLs is inhibited by increasing numbers of peptide-pulsed 221A2.1 targets. Two CTL lines at $1x10^5$ cells per well were incubated for 5 hours with $1x10^4$ ^{51}Cr labelled SW403 cells/well and varying numbers of 221A2.1 cells pulsed overnight with 10 ug/ml CEA.687 peptide (line 15, closed circle; line 42, closed square) or a nonspecific peptide, in this case HBV.18 (line 15, open circle; line 42, open square).

Figures 5a and 5b. Recognition of MAGE3.159 peptide-pulsed targets and tumor targets by peptide specific CTLs in the IFN γ ELISA. Figure 5a shows CTLs from individual wells screened against 221A2.1 without peptide (hatched bars) or 221A2.1 pulsed

overnight with 10 ug/ml peptide (solid bars). Figure 5b represents CTL recognition of 888mel (the negative, HLA mismatched tumor line; hatched bars) and 624mel, the positive tumor target line (solid bars).

Figures 6a and 6b. Recognition of MAGE3.160 peptide-pulsed targets and tumor targets by peptide specific CTLs in the IFN γ ELISA. Figure 6a shows CTLs from individual wells screened against 221A2.1 without peptide (hatched bars) or 221A2.1 pulsed overnight with 10 ug/ml peptide (solid bars). Figure 6b represents CTL recognition of 888mel (the negative, HLA mismatched tumor line; hatched bars) and 624mel, the positive tumor target line (solid bars).

Figure 7. Lysis of HER2/neu.369 peptide-pulsed targets and tumor targets by peptide specific CTLs. CTLs from one well of the primary induction cultures were tested against 221A2.1 (open bar), 221A2.1 pulsed with 10 ug/ml peptide overnight (black bar), HT29 (negative tumor targets, light gray bar) and SW403 (positive tumor targets, dark gray bar).

Figure 8a. CEA. 233V10 induced CTL are able to recognize peptide-pulsed 221A2.1 cells. CTLs were tested against 221A2.1 cells (hatched bars) and 221A2.1 peptide-pulsed cells (black bars).

Figure 8b. Expanded CEA. 233V10-specific CTLs from wells 4 and 10 were able to recognize the wildtype peptide and tumor targets as well as the analog. The CTLs were tested against 221A2.1 cells (hatched bars), 221A2.1 pulsed with CEA.233V10 (black bar), 221A2.1 pulsed with CEA.233 (crosshatched bar), HT29 (hatched), SW403 (white bar) and KATO III, a second CEA positive, A2 positive tumor target (gray bar).

Figure 9a. Recognition of peptide-pulsed target cells by CEA. 605V9 specific CTL. CTLs

from a primary induction with peptide CEA. 605V9 were tested for lytic activity against 221A2.1 without peptide (hatched bar) or 221A2.1 pulsed overnight with 10 ug/ml peptide (black bar). K562 cells were added to reduce lysis by natural killer (NK) cells.

Figure 9b. Expanded CEA.605V9 CTLs from wells 23 and 39 respectively recognize the wildtype peptide and tumor cells as well as the analog. The CTLs were tested in the IFNγ ELISA against 221A2.1 cells (hatched bars), 221A2.1 pulsed with CEA.605V9 (black bar), 221A2.1 pulsed with CEA.605 (crosshatched bar), HT29 (hatched), SW403 (white bar) and KATO III, a second CEA positive, A2 positive tumor target (gray bar).

Figure 10a. Recognition of peptide-pulsed target cells by CEA. 589V9 specific CTL. CTL from a primary induction with peptide CEA. 589V9 were tested for lytic activity against 221A2.1 without peptide (hatched bar) or 221A2.1 pulsed overnight with 10 ug/ml peptide (black bar). K562 cells were added to reduce lysis by NK cells.

Figures 10b and 10c. The expanded CTLs to CEA.589V9 (from wells 13 and 23 respectively) recognize the wildtype peptide. The CTLs were tested against 221A2.1 cells (open circle), 221A2.1 pulsed with CEA.589V9 (closed circle) and CEA.589 (open triangle).

Figure 11a. Peptide p53.139L2 induced CTL that recognized peptide-pulsed target cells. CTLs from individual wells were screened against 221A2.1 without peptide (hatched bars) or 221A2.1 pulsed overnight with 10 ug/ml peptide (solid bars). K562 cells were added to reduce lysis by NK cells.

Figures 11b and 11c. Expanded p53.139L2-specific CTL from wells 41 and 48 respectively, recognize the wildtype peptide. The targets were 221A2.1 cells (open circle), 221A2.1 pulsed with p53.139L2 (closed circle) and p53.139 (open triangle).

Figures 12a and 12b. Expanded p53.139L2-specific CTL, from wells 41 and 48 respectively, also recognize endogenously expressed p53. The cell lines tested were 221A2.1 cells (open circle), Saos-2 (open triangle), MCF-7 (closed triangle), SW480 (closed circle), and BT549 (closed square).

Figure 13a. CTLs induced with p53.139L2B3 peptide recognize peptide-pulsed 221A2.1 targets. CTLs from individual wells were screened against 221A2.1 without peptide (hatched bars) or 221A2.1 pulsed overnight with 10 ug/ml peptide (solid bars). K562 cells were added to reduce lysis by NK cells.

Figure 13b. Expanded p53.139L2B3 CTLs from well 42 recognize the wildtype peptide and tumor cells as well as the analog. The CTLs were tested in the IFNγ ELISA against 221A2.1 cells (hatched bars), 221A2.1 pulsed with p53.139L2B3 (black bar), 221A2.1 pulsed with p53.139 (crosshatched bar), Saos-2 (hatched) and BT549, a p53 positive, A2 positive tumor target (gray bar).

Figure 14a. CTLs induced with p53.149M2 peptide recognize 221A2.1 targets pulsed with the analog and wildtype peptides. CTLs from individual wells were screened against 221A2.1 without peptide (hatched bars) or 221A2.1 pulsed overnight with 10 ug/ml p53.149M2 (black bars) or p53.149. K562 cells were added to reduce lysis by NK cells.

Figure 14b. Expanded p53.149M2 CTLs from well 40 recognize the wildtype peptide and tumor cells as well as the analog. The CTLs were tested in the ^{51}Cr release assay against 221A2.1 cells (open circles), 221A2.1 pulsed with p53.149M2 (closed circles), 221A2.1 pulsed with p53.149 (open triangles), Saos-2, the p53 negative line (open squares) and Saos-2/175, a p53 positive, A2 positive transfected tumor target (closed squares).

Table I. Crossbinding data of 14 wild type CEA A*0201 binders.

Source	AA	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound
CEA.24	9	LLTFWNPPT	179	1720	67	755	--	2
CEA.78	9	QIIGYVIGT	313	148	106	100	150	5
CEA.233	10	VLYGPDAPTI	128	606	270	804	--	2
CEA.354	10	YLWWVNNQSL	26	108	26	487	67	5
CEA.411	10	VLYGPDDPTI	294	358	476	7400	--	3
CEA.432	9	NLSLSCHAA	455	2867	1449	18500	--	1
CEA.532	10	YLWWVNGQSL	33	331	21	2056	286	4
CEA.569	9	YVCGIQNSV	98	358	159	80	181	5
CEA.589	9	VLYGPDTPI	200	878	53	638	--	2
CEA.605	9	YLSGANLNL	28	165	2.4	804	--	3
CEA.687	9	ATVGIMIGV	36	8.8	20	11	0.80	5
CEA.690	10	GIMIGVLVGV	64	205	31	142	500	5
CEA.691	9	IMIGVLVGV	69	62	13	106	89	5
CEA.691	10	IMIGVLVGVA	227	68	44	726	1509	3

-- indicates binding affinity ≥10,000nM.

Table II. Immunogenicity of 6 wild type CEA crossbinders.

Source	AA	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound	CTL Wild-type [1]	CTL Tumor
CEA.78	9	QIIGYVIGT	313	148	106	100	151	5	0/3	
CEA.354	10	YLWWVNNQSL	26	108	26	487	333	5	1/2	0/1
CEA.569	9	YVCGIQNSV	98	358	159	80	182	5	1/2	0/1
.A.605	9	YLSGANLNL	28	165	2.4	804	-- [2]	3	2/2	1/2
CEA.687	9	ATVGIMIGV	36	8.8	20	11	0.80	5	1/1	1/1
CEA.691	9	IMIGVLVGV	69	62	13	106	89	5	8/8	4/7

1) Number of donors yielding a positive response/total tested.

2) -- indicates binding affinity ≥10,000nM.

Table III. Crossbinding data of 14 wild type p53 A*0201 binders.

Source	AA	Disclosed	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound
p53.24	9	Melief	KLLPENNVL	313	1955	--	1194	--	1
p53.25	11	Sherman	LLPENNVLSPL	19	6.2	4.5	12	1702	4
p53.65	10	Melief	RMPEAAPPVA	78	102	13	841	--	3
p53.65	9	Melief	RMPEAAPPV	119	23	22	70	--	4
p53.113	10	Melief	FLHSGTAKSV	357	179	15	4625	--	3
p53.132	9	Melief	KMFCQLAKT	333	33	18	106	--	4
p53.135	9		CQLAKTCPV	208	43	143	90	--	4
p53.136	8		QLAKTCPV	455	--	100	2643	1067	2
p53.164	9	Melief	KQSQHMTEV	500	130	170	285	--	4
p53.187	11	Melief/Sherman	GLAPPQHLIRV	79	39	11	55	--	4
p53.193	11	Melief	HLIRVEGNLRV	385	1387	83	1194	1778	2
p53.229	9	Sherman	CTTIHYNYM	278	287	2564	561	181	3
p53.263	10	Melief	NLLGRNSFEV	217	--	2500	881	--	1
p53.264	9	Melief	LLGRNSFEV	85	358	37	206	--	4

-- indicates binding affinity ≥10,000nM.

Table IV. Immunogenicity of a wild-type p53 crossbinder.

Source	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound	CTL Wild-type [1]	CTL Tumor
p53.135	CQLAKTCPV	208	43	143	90	-- [2]	4	1/4	0/1

1) Number of donors yielding a positive response/total tested.
2) -- indicates binding affinity ≥10,000nM.

Table V. Crossbinding data of 19 wild-type MAGE2/3 A*0201 binders.

Source	AA	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound
MAGE2.112	9	KMVELVHFL	38	15	9.1	49	364	5
MAGE2.112	10	KMVELVHFLL	23	39	127	9.0	2667	4
MAGE2.112	11	KMVELVHFLLL	5.0	45	63	109	7692	4
MAGE2.153	9	KASEYLQLV	152	116	17	185	4878	4
MAGE2.157	10	YLQLVFGIEV	50	165	345	370	9302	4
MAGE2.160	10	LVFGIEVVEV	357	21	44	29	8.0	5
MAGE2.220	9	KIWEELSML	167	642	175	29	--	3
MAGE2.271	9	FLWGPRALI	238	96	137	1542	95	4
MAGE2.277	10	ALIETSYVKV	500	729	125	1947	3077	2
MAGE2/3.44	10	TLVEVTLGEV	67	39	4.3	218	33	5
MAGE3.112	9	KVAELVHFL	68	29	14	168	17	5
MAGE3.112	10	KVAELVHFLL	54	36	217	206	11	5
MAGE3.159	11	QLVFGIELMEV	7.9	74	217	185	267	5
MAGE3.160	10	LVFGIELMEV	29	20	7.7	29	14	5
MAGE3.174	11	HLYIFATCLGL	56	741	769	--	4494	1
MAGE3.176	9	YIFATCLGL	185	45	37	1028	222	4
MAGE3.195	11	IMPKAGLLIIV	20	226	15	176	--	4
MAGE3.220	9	KIWEELSVL	333	391	2381	308	--	3
MAGE3.271	9	FLWGPRALV	31	43	14	336	40	5

-- indicates binding affinity ≥10,000nM.

Table VI. Immunogenicity of 13 wild-type MAGE2/3 crossbinders.

Source	AA	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound	CTL Wild-type [1]	CTL Tumor
MAGE2.112	9	KMVELVHFL	9.8	25	17	123	2353	4	1/1	0/1
MAGE2.112	10	KMVELVHFLL	23	39	127	9.0	2667	4	1/1	0/1
MAGE2.112	11	KMVELVHFLLL	5.0	45	63	109	7692	4	1/1	0/1
MAGE2.153	9	KASEYLQLV	152	116	17	185	4878	4	2/4	0/2
MAGE2.157	10	YLQLVFGIEV	50	165	345	370	9302	4	3/3	1/3
MAGE2.160	10	LVFGIEVVEV	357	20	43	28	8.0	5	4/4	0/3
MAGE3.112	9	KVAELVHFL	68	29	14	168	17	5	3/4	3/4
MAGE3.112	10	KVAELVHFLL	54	36	217	206	11	5	0/1	0/1
MAGE3.159	11	QLVFGIELMEV	7.9	74	217	185	267	5	3/3	1/3[2]
MAGE3.160	10	LVFGIELMEV	29	20	7.7	28	14	5	4/4	1/4[2]
MAGE3.195	11	IMPKAGLLIIV	20	226	14	176	--[3]	4	3/4	0/3
MAGE3.220	9	KIWEELSVL	357	391	2381	308	--	3	3/4	0/3
MAGE3.271	9	FLWGPRALV	31	43	14	336	40	5	4/4	2/4

1) Indicates the number of donors positive over the total number of donors tested.

2) A positive result was seen after the second restim.

3) -- indicates binding affinity ≥10,000nM.

Table VII. Crossbinding data of 20 wild-type Her2/neu A*0201 binders.

Source	AA	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound
Her2/neu.5	9	ALCRWGLLL	100	--	278	--	--	2
Her2/neu.5	10	ALCRWGLLLA	139	1955	12	1947	2500	2
Her2/neu.48	9	HLYQGCQVV	139	307	13	514	1143	3
Her2/neu.106	9	QLFEDNYAL	17	226	11	463	2105	4
Her2/neu.106	10	QLFEDNYALA	357	662	9.1	218	74	4
Her2/neu.144	10	SLTEILKGGV	238	--	22	--	--	2
Her2/neu.153	9	VLIQRNPQL	23	3909	3.3	1057	--	2
Her2/neu.369	9	KIFGSLAFL	36	9.0	19	23	3333	4
Her2/neu.435	9	ILHNGAYSL	75	358	100	569	--	3
Her2/neu.466	9	ALIHHNTHL	278	1265	10	1762	--	2
Her2/neu.508	9	GLACHQLCA	417	--	127	--	9091	2
Her2/neu.653	9	SIISAVVGI	69	524	35	285	148	4
Her2/neu.665	9	VVLGVVFGI	14	--	2500	430	2000	2
Her2/neu.689	9	RLLQETELV	21	--	625	34	--	2
Her2/neu.767	9	ILDEAYVMA	238	--	4167	3083	--	1
Her2/neu.773	10	VMAGVGSPYV	200	391	13	3700	--	3
Her2/neu.789	9	CLTSTVQLV	208	457	6.7	308	8000	4
Her2/neu.799	9	QLMPYGCLL	217	977	114	712	--	2
Her2/neu.952	10	YMIMVKCWMI	20	307	83	116	267	5
Her2/neu.952	9	YMIMVKCWM	217	--	625	2643	1000	1

-- indicates binding affinity ≥10,000nM.

Table VIII. Immunogenicity of 10 wild-type Her2/neu peptides.

Source	AA	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound	CTL Wild-type [1]	CTL Tumor [1]	CTL Wild-type [2]	CTL Tumor [2]
Her2/neu.5	9	ALCRWGLLL	100	– [3]	278	–	–	2	2/2	2/2		
Her2/neu.48	9	HLYQGCQVV	139	307	13	514	1143	3	1/2	0/2	2/2	1/2
Her2/neu.106	9	QLFEDNYAL	17	226	11	463	2105	4	0/2	0/2		
Her2/neu.106	10	QLFEDNYALA	357	662	9.1	218	74	4	0/2	0/2		
Her2/neu.369	9	KIFGSLAFL	36	9.0	19	23	3333	4	6/7	4/7	2/2	2/2
Her2/neu.435	9	ILHNGAYSL	75	358	100	569	–	3	3/3	1/3	2/2	2/2
Her2/neu.653	9	SIISAVVGI	69	524	35	285	148	4	0/3			
Her2/neu.773	10	VMAGVGSPYV	200	391	13	3700	–	3	1/2	0/2	1/2	1/2
Her2/neu.789	9	CLTSTVQLV	208	457	6.7	308	8000	4	1/4	0/4	1/2	
Her2/neu.952	10	YMIMVKCWMI	20	307	83	116	267	5	0/1	0/1	2/2	2/2

1) Number of donors yielding a positive response/total tested.
2) Kiessling data from ovarian cancer patients.
3) – indicates binding affinity ≥10,000nM.

Table IX. Crossbinding data of 9 fixed CEA peptides. [1]

Source	AA	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound
CEA.24	9	LLTFWNPPT	179	1720	67	755	-- [2]	2
CEA.24M2V9	9	LMTFWNPPV	4.5	782	7.7	34	3333	3
CEA.24V9	9	LLTFWNPPV	16	307	26	56	952	4
CEA.78	9	QIIGYVIGT	313	148	106	100	150	5
CEA.78L2V9	9	QLIGYVIGV	9.4	5.9	2.3	21	2.3	5
CEA.233	10	VLYGPDAPTI	128	606	270	804	--	2
CEA.233V10	10	VLYGPDAPTV	26	430	16	206	952	4
CEA.411	10	VLYGPDDPTI	294	358	476	7400	--	3
CEA.411V10	10	VLYGPDDPTV	161	105	91	2467	--	3
CEA.569	9	YVCGIQNSV	98	358	159	80	181	5
CEA.569L2	9	YLCGIQNSV	50	24	12	31	3478	4
CEA.589	9	VLYGPDTPI	200	878	53	638	--	2
CEA.589V9	9	VLYGPDTPV	20	165	91	154	9756	4
CEA.605	9	YLSGANLNL	28	165	2.4	804	--	3
CEA.605V9	9	YLSGANLNV	73	13	13	80	1600	4
CEA.687	9	ATVGIMIGV	36	8.8	20	11	0.80	5
CEA.687L2	9	ALVGIMIGV	10	63	31	100	102	5
CEA.691	9	IMIGVLVGV	69	62	13	106	89	5
CEA.691L2	9	ILIGVLVGV	22	8.0	3.2	16	160	5

1) Wild-type peptides presented for reference purposes.
2) -- indicates binding affinity ≥10,000nM.

Table X. Immunogenicity of 4 fixed CEA crossbinders.

Source	AA	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound	CTL Peptide [1]	CTL Wild-type	CTL Tumor
CEA.24	9	LLTFWNPPT	179	1720	67	755	– [2]	2		0/1	0/1
CEA.24V9	9	LLTFWNPPV	16	307	26	56	952	4	1/1		1/1
CEA.233	10	VLYGPDAPTI	128	606	270	804	–	2		2/4	0/3
CEA.233V10	10	VLYGPDAPTV	26	430	16	206	952	4	3/4	2/2	1/4
CEA.589	9	VLYGPDTPI	200	878	53	638	–	2		1/1	0/1
CEA.589V9	9	VLYGPDTPV	20	165	91	154	9756	4	2/2	2/2	0/2
CEA.605	9	YLSGANLNL	28	165	2.4	804	–	3		2/2	1/2
CEA.605V9	9	YLSGANLNV	73	13	13	80	1600	4	4/4	3/4	1/4

1) Number of donors yielding a positive response/total tested.

2) – indicates binding affinity ≥10,000nM.

Table XI. A*0201 binding of 20 fixed p53 peptides.

Source	AA	Sequence	A*0201 nM
p53.24	9	KLLPENNVL	313
p53.24V9	9	KLLPENNVV	385
p53.25	11	LLPENNVLSPL	19
p53.25V9	11	LLPENNVLSPV	39
p53.65	9	RMPEAAPPV	119
p53.65L2	9	RLPEAAPPV	78
p53.65	10	RMPEAAPPVA	78
p53.65L2V10	10	RLPEAAPPVV	143
p53.65M2V10	10	RMPEAAPPVV	54
p53.69	8	AAPPVAPA	5000
p53.69L2V8	8	ALPPVAPV	217
p53.101	11	KTYQGSYGFRL	1786
p53.101L2V11	11	KLYQGSYGFRV	81
p53.113	11	FLHSGTAKSVT	5000
p53.113V11	11	FLHSGTAKSVV	1220
p53.129	9	ALNKMFCQL	735
p53.129V9	9	ALNKMFCQV	75
p53.129B7V9	9	ALNKMFBQV	192
p53.129	10	ALNKMFCQLA	1316
p53.129V10	10	ALNKMFCQLV	217
p53.132	9	KMFCQLAKT	333
p53.132V9	9	KMFCQLAKV	33
p53.132B4V9	9	KMFBQLAKV	125
p53.132L2V9	9	KLFCQLAKV	98
p53.135	9	CQLAKTCPV	208
p53.135L2	9	CLLAKTCPV	125
p53.135B1B7	9	BQLAKTBPV	102
p53.135B1L2B7	9	BLLAKTBPV	46
p53.139	9	KTCPVQLWV	725
p53.139L2	9	KLCPVQLWV	122
p53.139L2B3	9	KLBPVQLWV	46
p53.149	9	STPPPGTRV	909
p53.149M2	9	SMPPPGTRV	172
p53.149L2	9	SLPPPGTRV	122
p53.164	9	KQSQHMTEV	500
p53.164L2	9	KLSQHMTEV	122
p53.216	10	VVVPYEPPEV	617
p53.216L2	10	VLVPYEPPEV	89
p53.229	9	CTTIHYNYM	278
p53.229L2V9	9	CLTIHYNYV	263
p53.229B1L2V9	9	BLTIHYNYV	116
p53.236	8	YMCNSSCM	4546
p53.236L2M8	8	YLCNSSCV	–
p53.236	11	YMCNSSCMGGM	667
p53.236L2M11	11	YLCNSSCMGGV	22
p53.255	11	ITLEDSSGNLL	1563
p53.255L2V11	11	ILLEDSSGNLV	33
p53.256	10	TLEDSSGNLL	1667
p53.256V10	10	TLEDSSGNLV	4167

– indicates binding affinity ≥10,000nM.

Table XII. Crossbinding data of 12 fixed p53 A*0201 peptides.

Source	AA	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound
p53.69	8	AAPPVAPA	5000	1536	1177	1233	4706	0
p53.69L2V8	8	ALPPVAPV	217	7167	500	285	67	4
p53.101	11	KTYQGSYGFRL	1786	896	–	514	615	0
p53.101L2V11	11	KLYQGSYGFRV	81	48	24	116	–	4
p53.129	9	ALNKMFCQL	735	391	19	73	–	3
p53.129V9	9	ALNKMFCQV	75	165	7.7	15	–	4
p53.129B7V9	9	ALNKMFBQV	192	391	23	49	–	4
p53.129	10	ALNKMFCQLA	1316	1075	71	4625	–	1
p53.129V10	10	ALNKMFCQLV	217	287	71	7400	–	3
p53.132	9	KMFCQLAKT	333	33	18	106	–	4
p53.132V9	9	KMFCQLAKV	33	8.4	7.7	15	–	4
p53.132B4V9	9	KMFBQLAKV	125	13	9.1	37	8889	4
p53.132L2V9	9	KLFCQLAKV	98	3.6	3.4	10	1270	4
p53.135	9	CQLAKTCPV	208	43	143	90	–	4
p53.135L2	9	CLLAKTCPV	125	506	67	370	–	3
p53.135B1B7	9	BQLAKTBPV	102	71	15	67	–	4
p53.135B1L2B7	9	BLLAKTBPV	46	119	7.7	64	–	4
p53.139	9	KTCPVQLWV	725	606	217	15	–	2
p53.139L2	9	KLCPVQLWV	122	239	29	23	–	4
p53.139L2B3	9	KLBPVQLWV	46	29	19	31	–	4
p53.149	9	STPPPGTRV	909	1162	1031	–	129	1
p53.149M2	9	SMPPPGTRV	172	215	13	425	667	4
p53.149L2	9	SLPPPGTRV	122	226	13	9250	140	4
p53.164	9	KQSQHMTEV	500	130	170	285	–	4
p53.164L2	9	KLSQHMTEV	122	94	35	46	–	4
p53.216	10	VVVPYEPPEV	617	1870	455	1194	–	1
p53.216L2	10	VLVPYEPPEV	89	391	71	2056	–	3
p53.236	11	YMCNSSCMGGM	667	391	67	974	5333	2
p53.236L2M11	11	YLCNSSCMGGV	22	13	3.6	18	1569	4
p53.255	11	ITLEDSSGNLL	1563	1265	2857	507	6667	0
p53.255L2V11	11	ILLEDSSGNLV	33	123	71	206	–	4

– indicates binding affinity ≥10,000nM.

Table XIII. Immunogenicity of 7 fixed p53 A*0201 peptides.

Source	AA	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound	CTL Peptide[1]	CTL Wild-type	CTL Tumor
p53.69	8	AAPPVAPA	5000	1536	1177	1233	4706	0			
p53.69L2V8	8	ALPPVAPV	217	7167	500	285	67	4	2/4	1/3	0/3
p53.129	9	ALNKMFCQL	735	391	19	73	--[2]	3			
p53.129V9	9	ALNKMFCQV	75	165	7.7	15	--	4	0/1		
p53.129B7V9	9	ALNKMFBQV	192	391	23	49	--	4	2/4	0/3	0/2
p53.132	9	KMFCQLAKT	333	33	18	106	--	4			
p53.132V9	9	KMFCQLAKV	33	8.4	7.7	15	--	4	1/3	0/2	0/2
p53.132B4V9	9	KMFBQLAKV	125	13	9.1	37	8889	4	5/5	0/4	0/4
p53.132L2V9	9	KLFCQLAKV	98	3.6	3.4	9.5	1270	4	2/3	1/3	0/3
p53.139	9	KTCPVQLWV	725	606	217	15	--	2			
p53.139L2	9	KLCPVQLWV	122	239	29	23	--	4	2/5	2/3	1/3
p53.139L2B3	9	KLBPVQLWV	45	29	19	31	--	4	3/4	2/3	1/2
p53.149	9	STPPPGTRV	909	1162	1031	--	129	1			
p53.149L2	9	SLPPPGTRV	122	226	13	9250	140	4	2/3	1/3	0/3
p53.149M2	9	SMPPPGTRV	172	215	13	425	667	4	2/4	2/4	2/4
p53.216	10	VVVPYEPPEV	617	1870	455	1194	--	1			
p53.216L2	10	VLVPYEPPEV	89	391	71	2056	--	3	1/1	1/1	
p53.255	11	ITLEDSSGNLL	1563	1265	2857	507	6667	0			
p53.255L2V11	11	ILLEDSSGNLV	33	123	71	206	--	4	1/3	0/3	0/2

1) Number of donors yielding a positive response/total tested.
2) -- indicates binding affinity ≥10,000nM.

Table XIV. Crossbinding data of 5 fixed MAGE2/3 A*0201 peptides.

Source	AA	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound
MAGE3.112	9	KVAELVHFL	69	29	14	168	17	5
MAGE3.112L2	9	KLAELVHFL	20	6.0	5.9	12	400	5
MAGE3.112M2	9	KMAELVHFL	24	6.7	7.7	26	286	5
MAGE3.112L2V9	9	KLAELVHFV	14	13	22	15	73	5
MAGE3.112M2V9	9	KMAELVHFV	26	17	46	39	170	5
MAGE3.220	9	KIWEELSVL	333	391	2381	308	--	3
MAGE3.220L2V9	9	KLWEELSVV	11	165	20	15	--	4

-- indicates binding affinity ≥10,000nM.

Table XV. Crossbinding data of 6 fixed Her2/neu A*0201 peptides.

Source	AA	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound
Her2/neu.5	9	ALCRWGLLL	100	--	278	--	--	2
Her2/neu.5B3V9	9	ALBRWGLLV	18	33	4.2	285	--	4
Her2/neu.5M2B3V9	9	AMBRWGLLV	36	473	16	726	--	3
Her2/neu.153	9	VLIQRNPQL	23	3909	3.3	1057	--	2
Her2/neu.153V9	9	VLIQRNPQV	55	768	135	385	--	3
Her2/neu.369	9	KIFGSLAFL	36	9.0	19	23	3333	4
Her2/neu.369L2V9	9	KLFGSLAFV	5.8	7.5	19	17	1270	4
Her2/neu.653	9	SIISAVVGI	69	524	35	285	148	4
Her2/neu.653.L2V9	9	SLISAVVGV	7.1	10	16	20	110	5
Her2/neu.665	9	VVLGVVFGI	14	--	2500	430	2000	2
Her2/neu.665L2V9	9	VLLGVVFGV	2.4	19	14	6.0	8000	4
Her2/neu.952	10	YMIMVKCWMI	20	307	83	116	267	5
Her2/neu.952L2V10	10	YLIMVKCWMV	13	56	116	18	84	5
Her2/neu.952L2B7V10	10	YLIMVKBWMV	7.2	66	77	11	851	4

-- indicates binding affinity ≥10,000nM.

Table XVI. Immunogenicity of fixed Her2/neu peptides.

Source	Sequence	A*0201 nM	A*0202 nM	A*0203 nM	A*0206 nM	A*6802 nM	No. A2 Alleles Crossbound	CTL Peptide [1]	CTL Wild-type [1]	CTL Tumor [1]
Her2/neu.5	ALCRWGLLL	100	– [2]	278	–	–	2		2/2	2/2
Her2/neu.5B3V9	ALBRWGLLV	18	33	4.2	285	–	4	2/3	nt	0/3
Her2/neu.5M2V9	AMCRWGLLV	179	7167	63	128	–	3	1/2	nt	0/2
Her2/neu.952	YMIMVKCWMI	20	307	83	116	267	5		0/1	0/1
Her2/neu.952L2B7V10	YLIMVKBWMV	7.2	66	77	11	851	4	3/3	nt	0/3

1) Number of donors yielding a positive response/total tested.

2) – indicates binding affinity ≥10,000nM.

Figure 1.

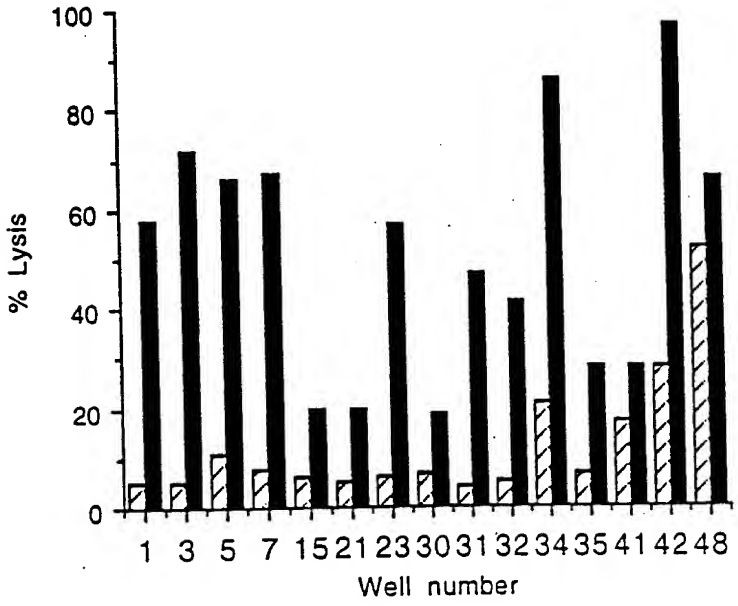


Figure 2a.

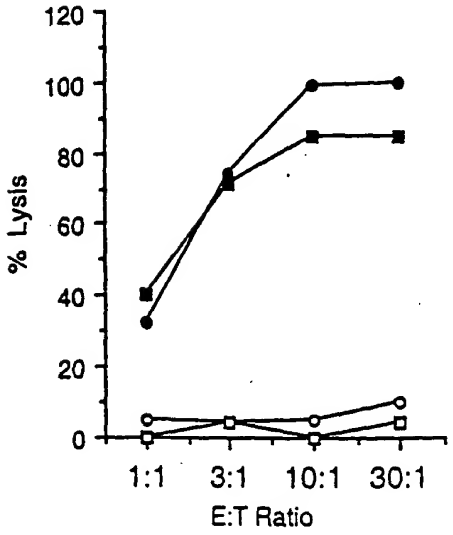


Figure 2b.

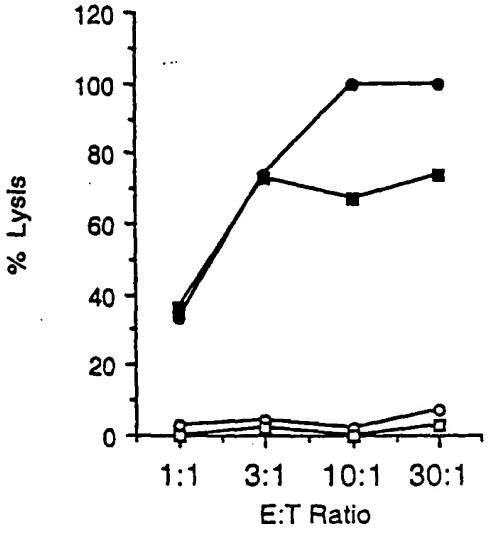


Figure 2c.

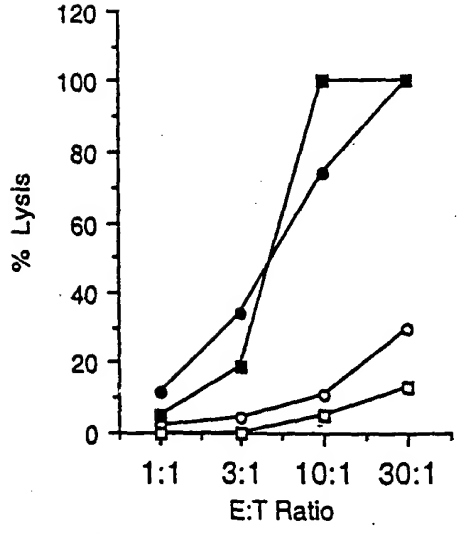


Figure 2d.

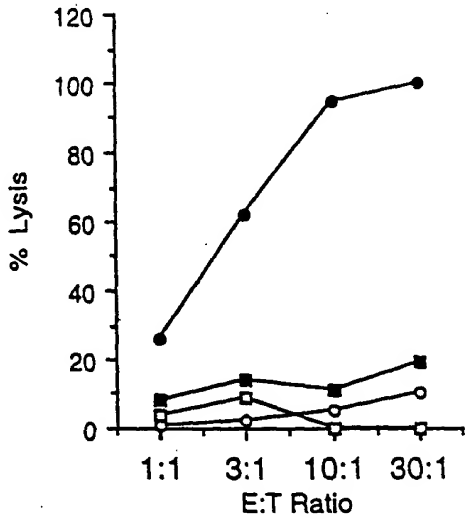


Figure 3.

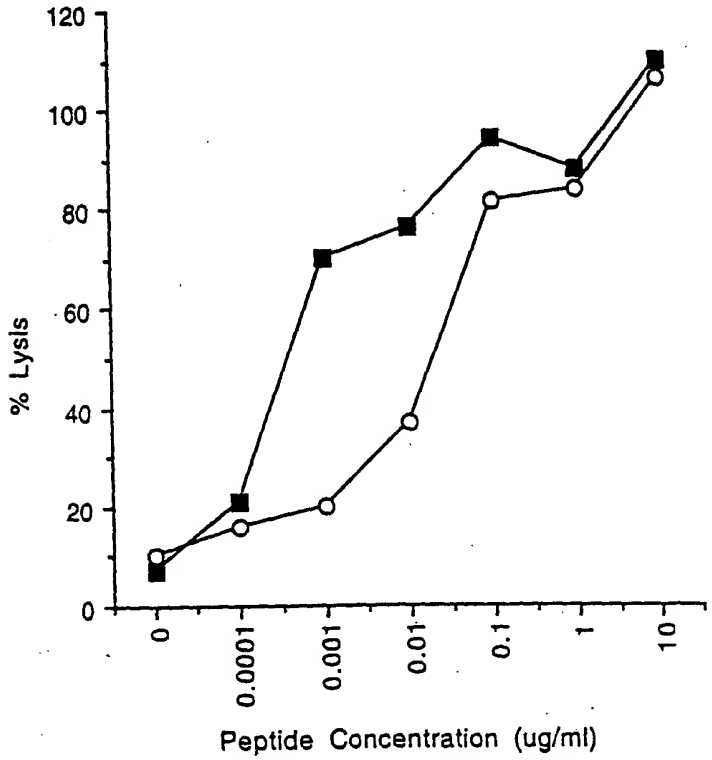
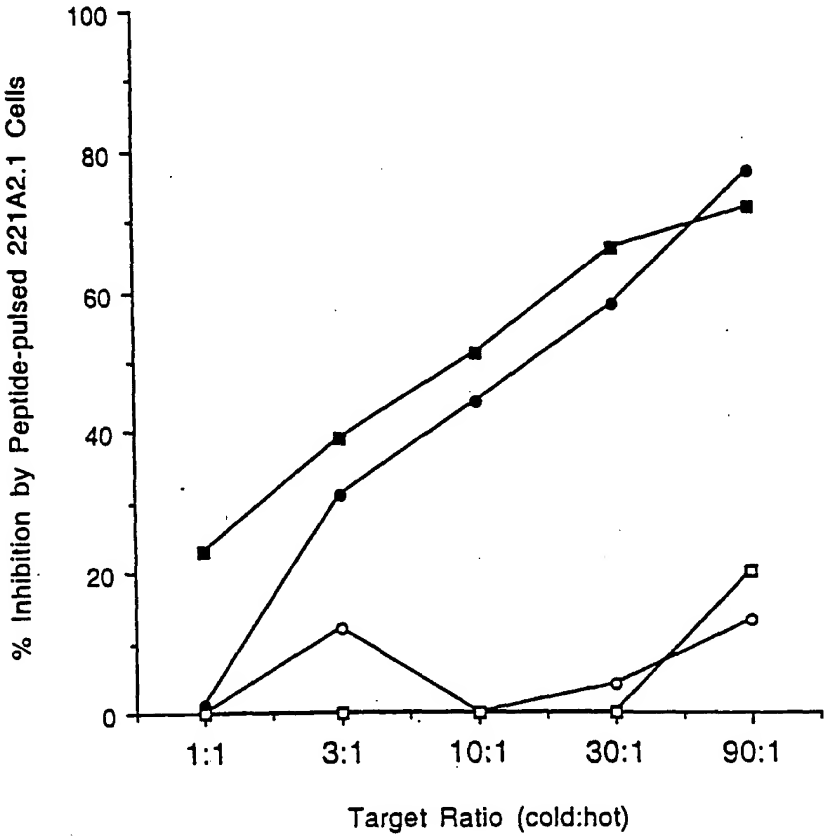


Figure 4.



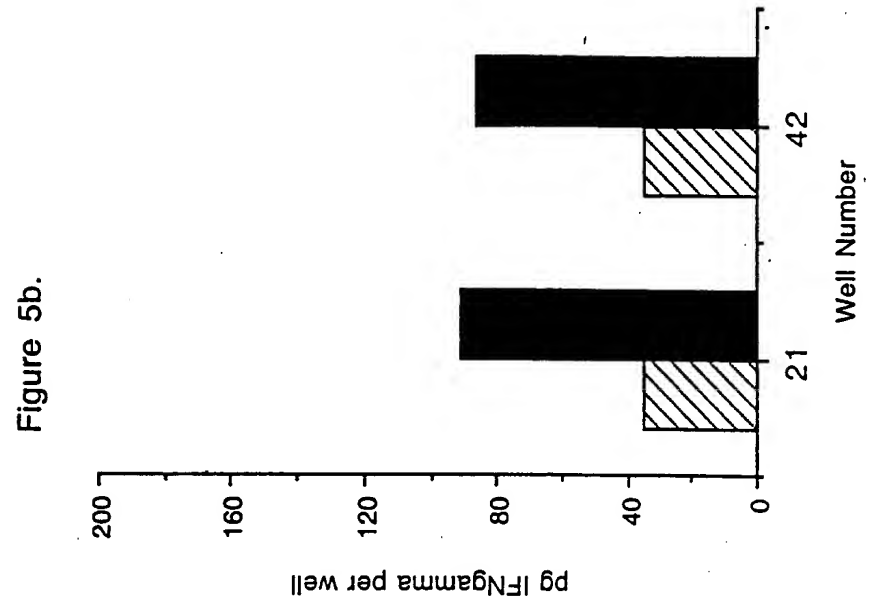


Figure 5b.

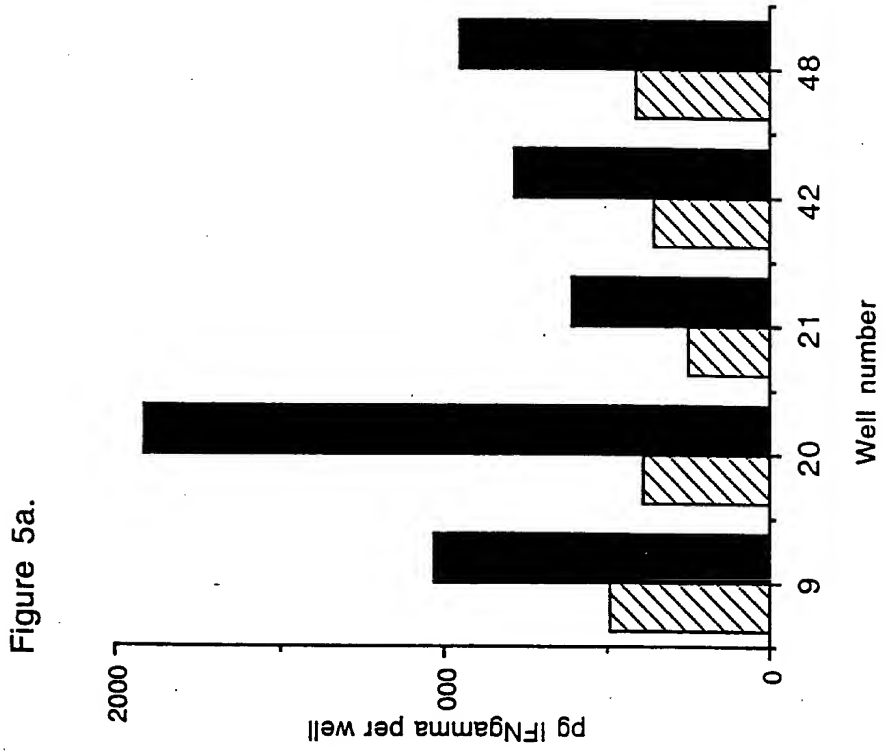
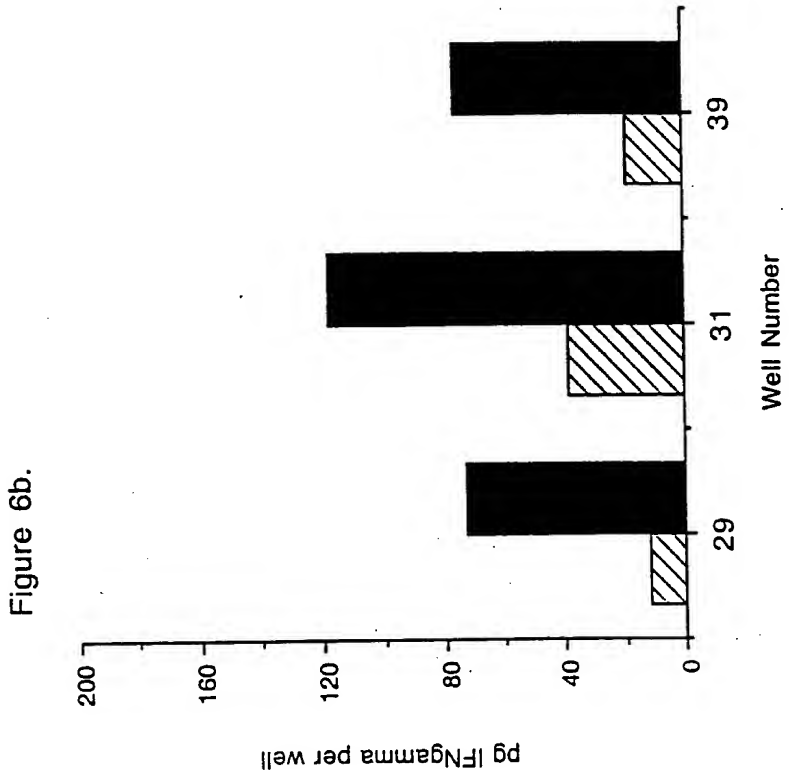
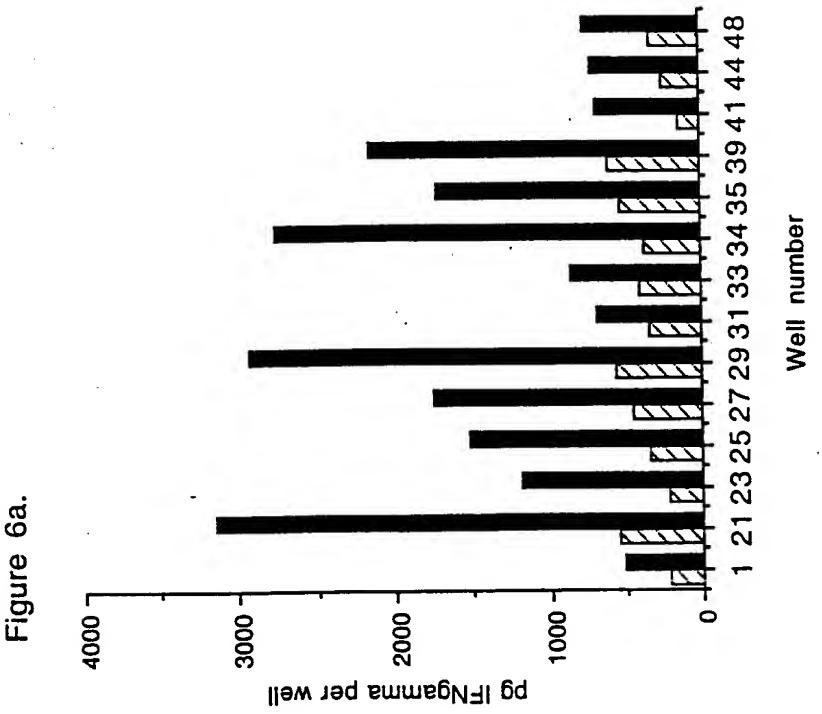


Figure 5a.





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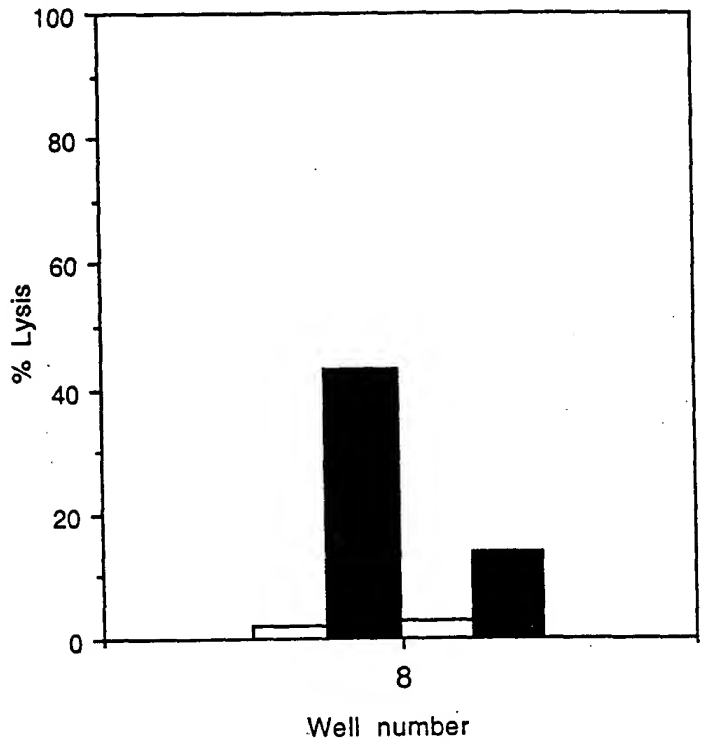

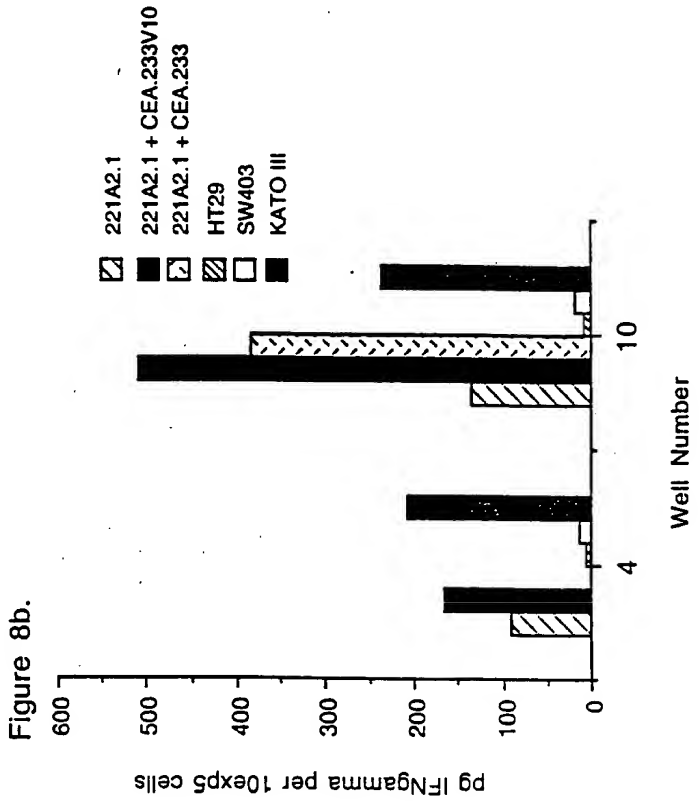

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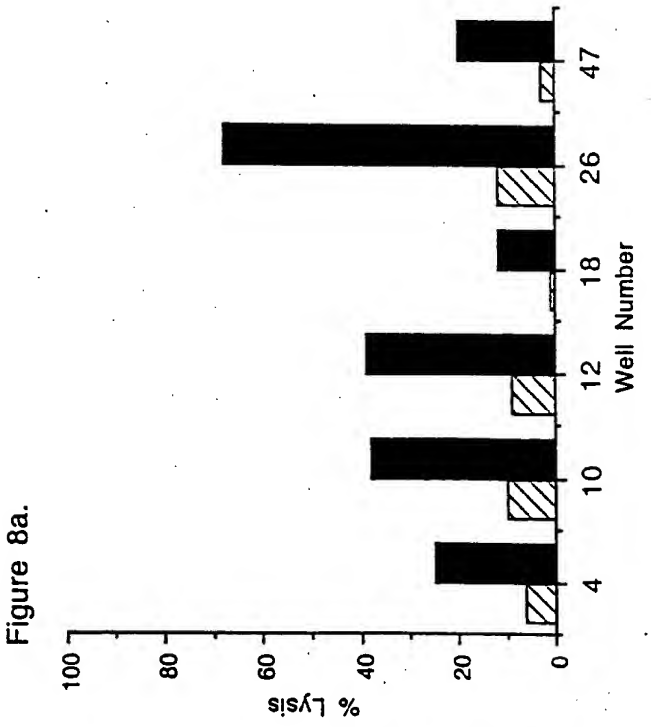


Figure 8a.

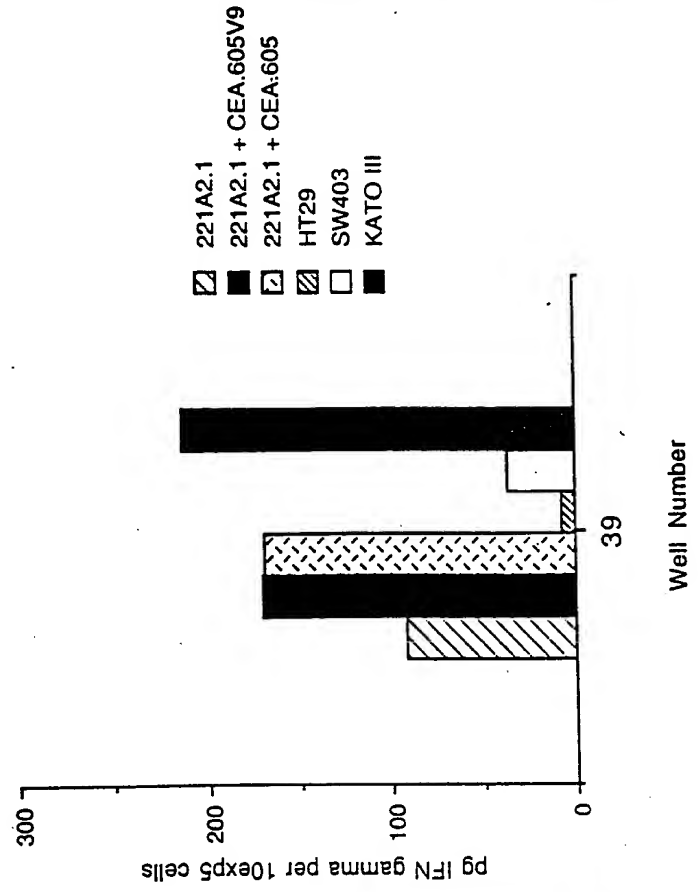


Figure 9b.

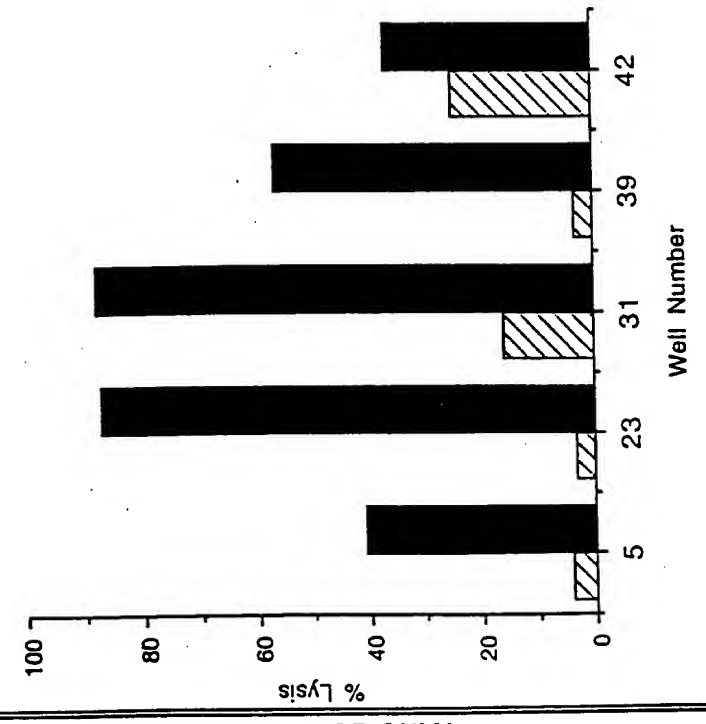
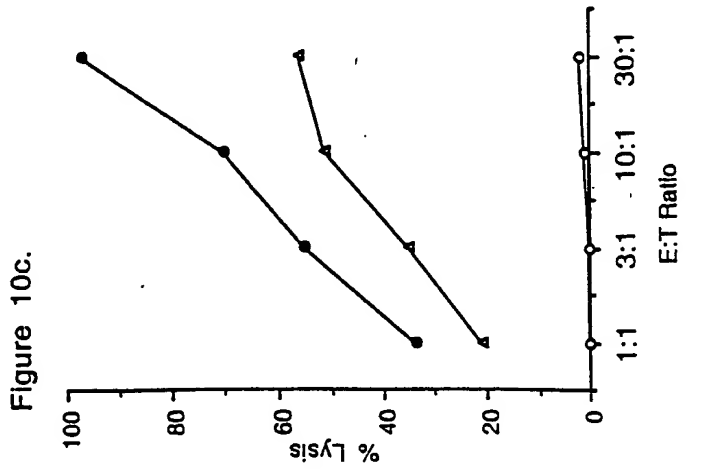
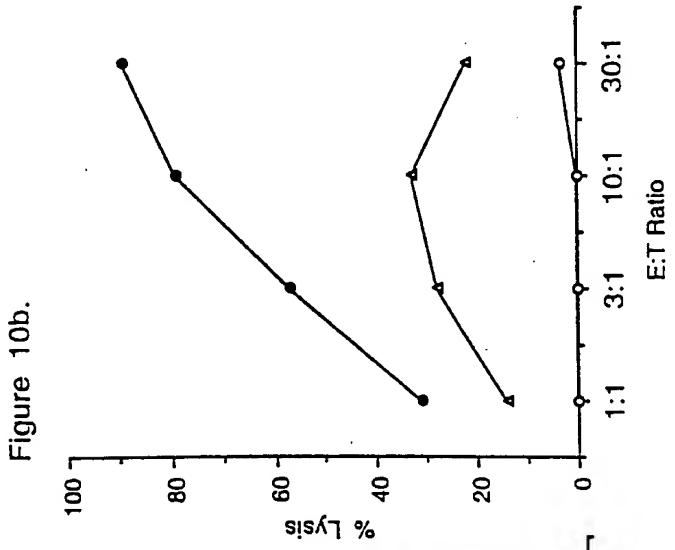
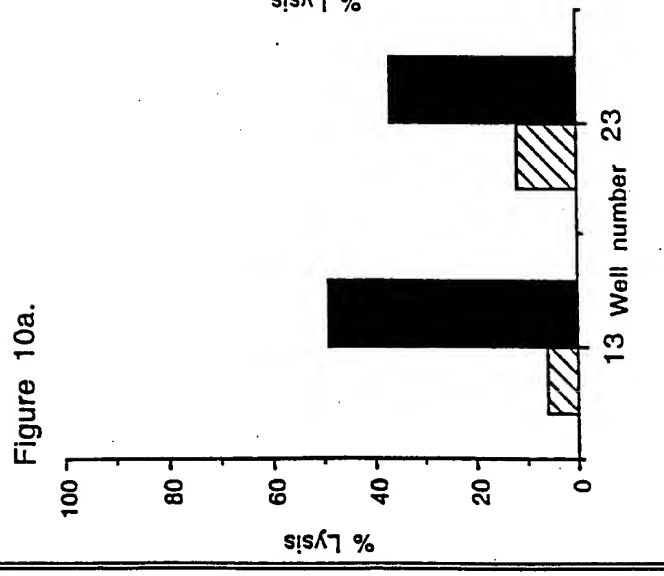


Figure 9a.







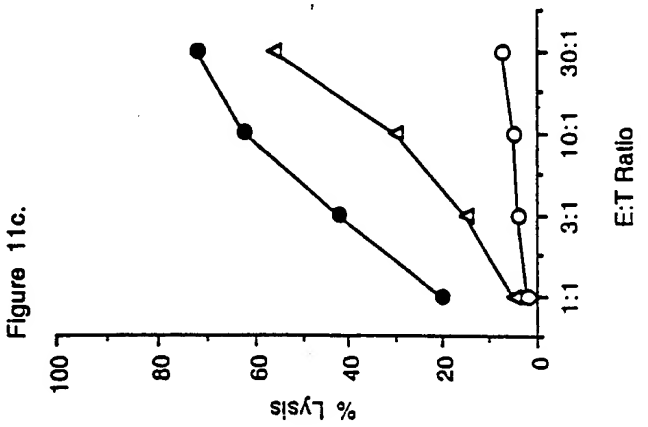


Figure 11c.

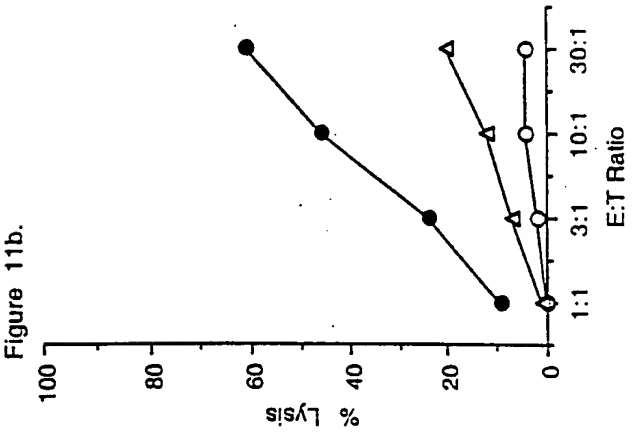


Figure 11b.

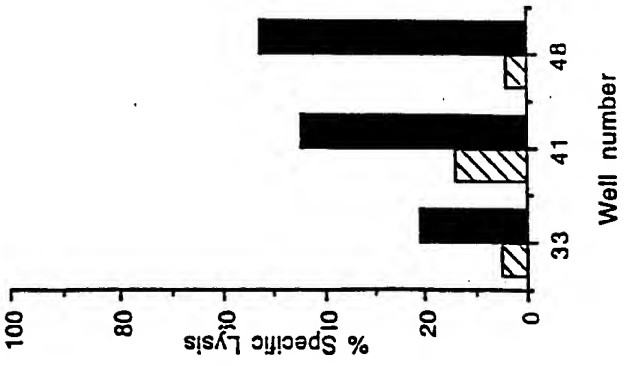


Figure 11a.

Figure 12a.

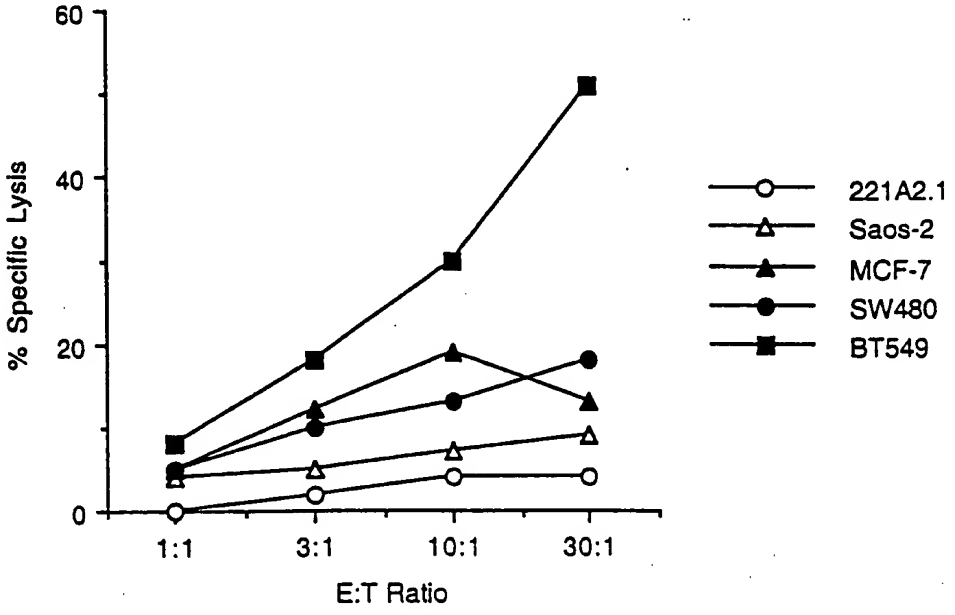


Figure 12b.

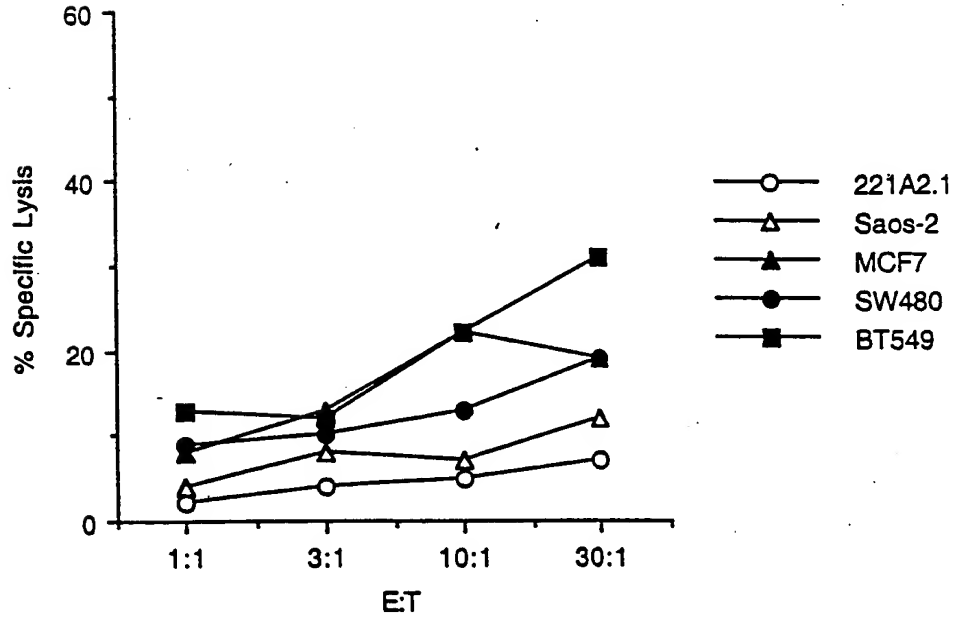


Figure 13a.

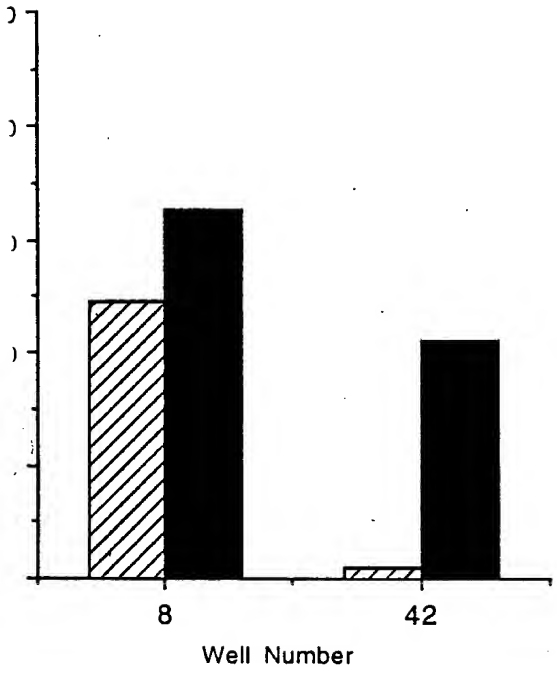


Figure 13b.

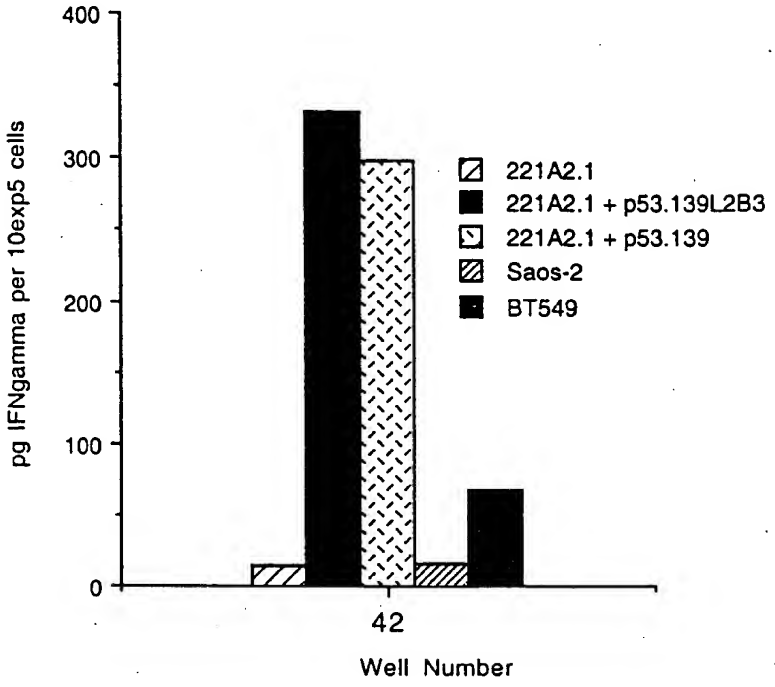


Figure 14a.

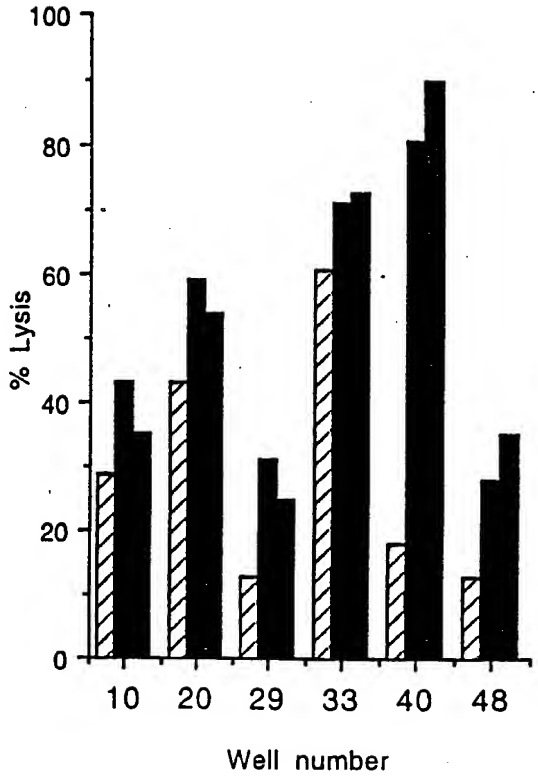
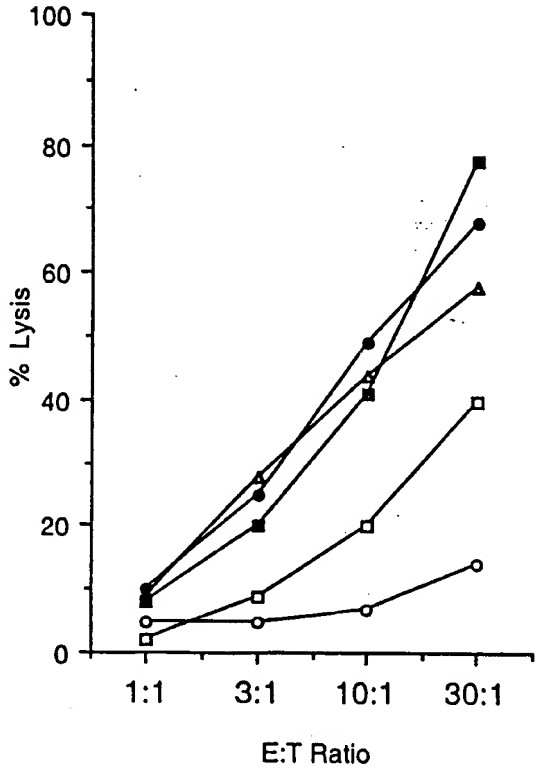


Figure 14b.



Docket No.: 399632001920	Atty: Kate H. Murashige
Serial No.: 09/189,702	Filing Date: November 10, 1998
Title: HLA BINDING PEPTIDES AND THEIR USES	
Date of Mailing: January 18, 2002 via First Class Mail	

Papers enclosed herewith:

1. Transmittal Form (1 page)
2. Fee Transmittal Form (1 page, 1 duplicate)
3. Amendment under 37 C.F. R. § 1.111 (12 pages) w exhibits A + B
4. Petition for Extension of Time (2 pages, 1 duplicate)
5. Exhibit A- Scott Southwood Bio.
6. Exhibit B- Declaration of Scott Southwood
   Tables I-XVI & Figures 1-14
7. (2) papers by Carmen Gianfrani, et al. and Keogh, et al.
8. Return Postcard

THE STAMP OF THE UNITED STATES PATENT AND TRADEMARK OFFICE HEREON WILL ACKNOWLEDGE RECEIPT VIA FIRST CLASS MAIL OF THE ABOVE DOCUMENTS